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UNITED STATES DISTRICT COURT
DISTRICT OF NEW JERSEY

JAZZ PHARMACEUTICALS, INC. and GENTIUM S.R.L., **Plaintiffs,** **v.** **ALMAJECT, INC., ALVOGEN, INC., and ALVOGEN PB RESEARCH AND DEVELOPMENT LLC,** **Defendants.**	**Civil Action No. _____** **COMPLAINT FOR PATENT INFRINGEMENT** **(Filed Electronically)**

Plaintiffs Jazz Pharmaceuticals, Inc. and Gentium S.r.l. (together, "Jazz Pharmaceuticals"), by their undersigned attorneys, for their Complaint against Defendants Almaject, Inc., Alvogen, Inc., and Alvogen PB Research and Development LLC (collectively, "Almaject" or "Defendants"), allege as follows:

Nature of the Action

1. This is an action for patent infringement under the patent laws of the United States, 35 U.S.C. §100, *et seq*., arising from Almaject's submission of Abbreviated New Drug Application ("ANDA") No. 216293 ("Almaject's ANDA") to the United States Food and Drug

Administration ("FDA") seeking approval to manufacture, use, import, distribute, offer to sell, and/or sell a generic version of Jazz Pharmaceuticals' Defitelio® (defibrotide sodium) drug product prior to the expiration of United States Patent Nos. 11,085,043 ("the '043 patent"), 11,236,328 ("the '328 patent") and 11,746,348 ("the '348 patent) (collectively "the patents-in-suit"), owned by Jazz Pharmaceuticals.

The Parties

2. Plaintiff Jazz Pharmaceuticals, Inc. is a corporation organized and existing under the laws of the State of Delaware, having a principal place of business at 3170 Porter Drive, Palo Alto, CA 94304.

3. Plaintiff Gentium S.r.l. is a corporation organized and existing under the laws of Italy, having a principal place of business at Piazza XX Settembre, 2, 22079 Villa Guardia CO, Italy.

4. On information and belief, Defendant Almaject, Inc. is a corporation organized and existing under the laws of the State of Delaware, having a principal place of business at 44 Whippany Road, Suite 300, Morristown, New Jersey 07960.

5. On information and belief, Defendant Alvogen, Inc. is a corporation organized and existing under the laws of the State of Delaware, having a principal place of business at 44 Whippany Road, Suite 300, Morristown, New Jersey 07960.

6. On information and belief, Defendant Alvogen PB Research and Development LLC is a corporation organized and existing under the laws of the State of Delaware, having a principal place of business at 10 Bloomfield Ave., Building B, Pine Brook, NJ 07058. On information and belief, Alvogen PB Research and Development LLC is the regulatory agent for Almaject, Inc.

7. On information and belief, Almaject, Inc. and Alvogen PB Research and Development LLC are wholly owned subsidiaries of Alvogen, Inc.

The Patents-in-Suit

8. On August 10, 2021, the United States Patent and Trademark Office ("USPTO") duly and lawfully issued the '043 patent, entitled, "Euglobulin-based Method for Determining the Biological Activity of Defibrotide." The face of the '043 patent identifies Terenzio Ignoni, Vijay Kumar, and Khalid Islam as the inventors. A copy of the '043 patent is attached hereto as Exhibit A.

9. On February 1, 2022, the USPTO duly and lawfully issued the '328 patent, entitled, "Euglobulin-based Method for Determining the Biological Activity of Defibrotide." The face of the '328 patent identifies Terenzio Ignoni, Vijay Kumar, and Khalid Islam as the inventors. A copy of the '328 patent is attached hereto as Exhibit B.

10. On September 5, 2023, the USPTO duly and lawfully issued the '348 patent, entitled, "Euglobulin-based Method for Determining the Biological Activity of Defibrotide." The face of the '348 patent identifies Terenzio Ignoni, Vijay Kumar, and Khalid Islam as the inventors. A copy of the '348 patent is attached hereto as Exhibit C.

The Defitelio® Drug Product

11. Jazz Pharmaceuticals holds an approved New Drug Application ("NDA") under Section 505(a) of the Federal Food Drug and Cosmetic Act ("FFDCA"), 21 U.S.C. § 355(a), for defibrotide sodium (NDA No. 208114), which it sells under the trademark Defitelio®. Defitelio® is an FDA-approved medication used for the treatment of adult and pediatric patients with hepatic veno-occlusive disease (VOD), also known as sinusoidal obstruction syndrome (SOS), with renal or pulmonary dysfunction following hematopoietic stem-cell transplantation (HSCT).

12. The claims of the patents-in-suit cover, *inter alia*, defibrotide formulations and methods of treating veno-occlusive disease with defibrotide formulations.

13. Pursuant to 21 U.S.C. § 355(b)(1) and attendant FDA regulations, the '043, '328, and '348 patents are listed in the FDA publication, "Approved Drug Products with Therapeutic Equivalence Evaluations" (the "Orange Book"), with respect to Defitelio®.

14. The labeling for Defitelio® instructs and encourages physicians, pharmacists, other healthcare workers, and patients to administer and/or use Defitelio® for the treatment of adult and pediatric patients with hepatic veno-occlusive disease (VOD), also known as sinusoidal obstruction syndrome (SOS), with renal or pulmonary dysfunction following hematopoietic stem-cell transplantation (HSCT).

15. The labeling for Defitelio® instructs and encourages physicians, pharmacists, other healthcare workers, and patients to administer and/or use Defitelio® according to one or more of the methods claimed in the patents-in-suit.

Jurisdiction and Venue

16. This Court has jurisdiction over the subject matter of this action pursuant to 28 U.S.C. §§ 1331, 1338(a), 2201, and 2202.

17. On information and belief, Almaject, Inc. derives substantial revenue from directly or indirectly selling generic pharmaceutical products and/or active pharmaceutical ingredient(s) used in generic pharmaceutical products sold throughout the United States, including in this Judicial District.

18. This Court has personal jurisdiction over Almaject, Inc. because, *inter alia*, it: (1) on information and belief, maintains a regular and established, physical place of business at 44 Whippany Road, Suite 300, Morristown, New Jersey 07960; and (2) maintains extensive and

systematic contacts with the State of New Jersey, including through the marketing, distribution, and/or sale of generic pharmaceutical drugs in New Jersey including through, directly or indirectly, Alvogen PB Research and Development LLC. On information and belief, Almaject, Inc. is registered with the State of New Jersey's Division of Revenue and Enterprise Services as a business operating in New Jersey under Business ID No. 0101049834. On information and belief, Almaject, Inc. is registered with the State of New Jersey's Department of Health as a drug manufacturer and wholesaler under Registration No. 5005555. By virtue of its physical presence in New Jersey, this Court has personal jurisdiction over Almaject, Inc. On information and belief, Almaject, Inc. purposefully has conducted and continues to conduct business in this Judicial District.

19. On information and belief, Alvogen, Inc. derives substantial revenue from directly or indirectly selling generic pharmaceutical products and/or active pharmaceutical ingredient(s) used in generic pharmaceutical products sold throughout the United States, including in this Judicial District.

20. This Court has personal jurisdiction over Alvogen, Inc. because, *inter alia*, it: (1) on information and belief, maintains a regular and established, physical place of business at 44 Whippany Road, Suite 300, Morristown, New Jersey 07960; and (2) maintains extensive and systematic contacts with the State of New Jersey, including through the marketing, distribution, and/or sale of generic pharmaceutical drugs in New Jersey including through, directly or indirectly, Almaject, Inc. and/or Alvogen PB Research and Development LLC. On information and belief, Alvogen, Inc. is registered with the State of New Jersey's Division of Revenue and Enterprise Services as a business operating in New Jersey under Business ID No. 0400326850. On information and belief, Alvogen, Inc. is registered with the State of New Jersey's Department

of Health as a drug manufacturer and wholesaler under Registration No. 5003865. By virtue of its physical presence in New Jersey, this Court has personal jurisdiction over Alvogen, Inc. On information and belief, Alvogen, Inc. purposefully has conducted and continues to conduct business in this Judicial District.

21. On information and belief, Alvogen PB Research and Development LLC derives substantial revenue from directly or indirectly selling generic pharmaceutical products and/or active pharmaceutical ingredient(s) used in generic pharmaceutical products sold throughout the United States, including in this Judicial District.

22. This Court has personal jurisdiction over Alvogen PB Research and Development LLC because, *inter alia*, it: (1) on information and belief, maintains a regular and established, physical place of business at 10 Bloomfield Ave, Building B, Pine Brook, NJ 07058; and (2) maintains extensive and systematic contacts with the State of New Jersey, including through the marketing, distribution, and/or sale of generic pharmaceutical drugs in New Jersey including through, directly or indirectly, Almaject, Inc. and/or Alvogen Inc. By virtue of its physical presence in New Jersey, this Court has personal jurisdiction over Alvogen PB Research and Development LLC. On information and belief, Alvogen PB Research and Development LLC purposefully has conducted and continues to conduct business in this Judicial District.

23. On information and belief, Defendants Almaject, Inc., Alvogen, Inc., and Alvogen PB Research and Development LLC are in the business of, among other things, manufacturing, marketing, importing, offering for sale, and selling pharmaceutical products, including generic drug products, throughout the United States, including in this Judicial District. On information and belief, Almaject, Inc., Alvogen, Inc., and Alvogen PB Research and

Development LLC also prepare and/or aid in the preparation and submission of ANDAs to the FDA, including Almaject's ANDA.

24. On information and belief, this Judicial District is a likely destination for the generic drug product described in Almaject's ANDA.

25. This Court also has personal jurisdiction over Almaject, Inc., Alvogen, Inc., and Alvogen PB Research and Development LLC because, *inter alia*, they have committed an act of patent infringement under 35 U.S.C. § 271(e)(2). On information and belief, Almaject, Inc., Alvogen, Inc., and Alvogen PB Research and Development LLC intend a future course of conduct that includes acts of patent infringement in New Jersey. These acts have led and will continue to lead to foreseeable harm and injury to Jazz Pharmaceuticals in New Jersey and in this Judicial District.

26. On information and belief, Almaject, Inc., Alvogen, Inc., and Alvogen PB Research and Development LLC work in privity and/or concert either directly or indirectly through one or more of their wholly owned subsidiaries with respect to the regulatory approval, manufacturing, use, importation, marketing, offer for sale, sale, and distribution of generic pharmaceutical products throughout the United States, including in this Judicial District.

27. On information and belief, each of Almaject, Inc., Alvogen, Inc., and Alvogen PB Research and Development LLC actively participated in the submission of Almaject's ANDA. On information and belief, Almaject, Inc., Alvogen, Inc., and Alvogen PB Research and Development LLC will work in privity and/or concert with one another and/or other related entities towards the regulatory approval, manufacturing, use, importation, marketing, offer for sale, sale, and distribution of generic pharmaceutical products, including the proposed product

described in Almaject's ANDA, throughout the United States, including in New Jersey and in this Judicial District, prior to the expiration of the patents-in-suit.

28. On information and belief, Almaject, Inc. intends to benefit directly if Almaject's ANDA is approved by participating in the manufacture, importation, distribution, and/or sale of the generic drug product that is the subject of Almaject's ANDA.

29. On information and belief, Alvogen, Inc. intends to benefit directly if Almaject's ANDA is approved by participating in the manufacture, importation, distribution, and/or sale of the generic drug product that is the subject of Almaject's ANDA.

30. On information and belief, Alvogen PB Research and Development LLC intends to benefit directly if Almaject's ANDA is approved by participating in the manufacture, importation, distribution, and/or sale of the generic drug product that is the subject Almaject's ANDA.

31. On information and belief, Almaject, Inc. and Alvogen PB Research and Development LLC act at the direction, and for the benefit, of Alvogen, Inc., and are controlled and/or dominated by Alvogen, Inc.

32. On information and belief, Defendants Almaject, Inc., Alvogen, Inc., and Alvogen PB Research and Development LLC act, operate, and/or hold themselves out to the public as a single integrated business.

33. On information and belief, Defendants Alvogen, Inc. and Alvogen PB Research and Development LLC have previously been sued in this District and have not challenged personal jurisdiction. *See, e.g.*, *Salix Pharmaceuticals, Inc. et al. v. Norwich Pharmaceuticals, Inc. et al.,* Civ. Action No. 1:24-cv-07140 (ESK)(AMD) (D.N.J.) (Alvogen, Inc. and Alvogen

PB Research and Development LLC); *Boehringer Ingelheim Pharmaceuticals Inc. et al. v. Alvogen, Inc.*, Civ. Action No. 2:23-cv-03911 (MEF)(JRA) (D.N.J.) (Alvogen, Inc.).

34. Venue is proper in this Judicial District pursuant to 28 U.S.C. §§ 1391 and/or 1400(b).

Acts Giving Rise To This Suit

35. Pursuant to Section 505 of the FFDCA, Almaject submitted Almaject's ANDA seeking approval to engage in the commercial manufacture, use, sale, or offer for sale in, or importation into, the United States of defibrotide sodium vials, 250 mg/2.5 mL ("Almaject's Proposed Product") before the patents-in-suit expire.

36. On information and belief, following FDA approval of Almaject's ANDA, Almaject will make, use, sell, or offer to sell Almaject's Proposed Product throughout the United States, or import such generic product into the United States.

37. On information and belief, in connection with the submission of Almaject's ANDA as described above, Almaject provided written certification to the FDA pursuant to Section 505 of the FFDCA, 21 U.S.C. § 355(j)(2)(A)(vii)(IV) ("Almaject's Paragraph IV Certification"), alleging that the claims of the patents-in-suit are invalid and/or will not be infringed by the activities described in Almaject's ANDA.

38. No earlier than March 4, 2025, Almaject sent notice of its Paragraph IV Certification to Jazz Pharmaceuticals ("Almaject's Notice Letter"). Almaject's Notice Letter alleged that the claims of the patents-in-suit are invalid and/or will not be infringed by the activities described in Almaject's ANDA. Almaject's Notice Letter also informed Jazz Pharmaceuticals that Almaject seeks approval to market Almaject's Proposed Product before the expiration of the patents-in-suit.

Count I for Infringement of the '043 Patent

39. Jazz Pharmaceuticals repeats and realleges the allegations of the preceding paragraphs as if fully set forth herein.

40. Almaject's submission of Almaject's ANDA to obtain approval to engage in the commercial manufacture, use, sale, or offer for sale in, or importation into, the United States of Almaject's Proposed Product, prior to the expiration of the '043 patent, constitutes infringement of one or more of the claims of that patent under 35 U.S.C. § 271(e)(2)(A), including at least claim 1.

41. Upon information and belief, Almaject's ANDA Product satisfies each and every element of at least claim 1 of the '043 patent.

42. There is a justiciable controversy between the parties hereto as to the infringement of the '043 patent.

43. Unless enjoined by this Court, upon FDA approval of Almaject's ANDA, Almaject, Inc., Alvogen, Inc., and Alvogen PB Research and Development LLC will infringe one or more claims of the '043 patent under 35 U.S.C. § 271(a), including at least claim 1, by making, using, offering to sell, selling, and/or importing Almaject's Proposed Product in or for the United States.

44. Unless enjoined by this Court, upon FDA approval of Almaject's ANDA, Almaject, Inc., Alvogen, Inc., and Alvogen PB Research and Development LLC will induce infringement of one or more claims of the '043 patent under 35 U.S.C. § 271(b), including at least claim 1, by making, using, offering to sell, selling, and/or importing Almaject's Proposed Product in or for the United States. On information and belief, upon FDA approval of Almaject's ANDA, Almaject, Inc., Alvogen, Inc., and Alvogen PB Research and Development

LLC will intentionally encourage acts of direct infringement with knowledge of the ’043 patent and knowledge that their acts are encouraging infringement.

45. Unless enjoined by this Court, upon FDA approval of Almaject’s ANDA, Almaject, Inc., Alvogen, Inc., and Alvogen PB Research and Development LLC will contributorily infringe one or more claims of the ’043 patent under 35 U.S.C. § 271(c), including at least claim 1, by making, using, offering to sell, selling, and/or importing Almaject’s Proposed Product in or for the United States. On information and belief, Almaject, Inc., Alvogen, Inc., and Alvogen PB Research and Development LLC have had and continue to have knowledge that Almaject’s Proposed Product is especially adapted for a use that infringes one or more claims of the ’043 patent and that there is no substantial non-infringing use for Almaject’s Proposed Product.

46. Jazz Pharmaceuticals will be substantially and irreparably damaged and harmed if Almaject, Inc., Alvogen, Inc., and Alvogen PB Research and Development LLC’s infringement of the ’043 patent is not enjoined.

47. Jazz Pharmaceuticals does not have an adequate remedy at law.

48. This case is an exceptional one, and Jazz Pharmaceuticals is entitled to an award of its reasonable attorneys’ fees under 35 U.S.C. § 285.

Count II for Infringement of the ’328 Patent

49. Jazz Pharmaceuticals repeats and realleges the allegations of the preceding paragraphs as if fully set forth herein.

50. Almaject’s submission of Almaject’s ANDA to obtain approval to engage in the commercial manufacture, use, sale, or offer for sale in, or importation into, the United States of Almaject’s Proposed Product, prior to the expiration of the ’328 patent, constitutes infringement

of one or more of the claims of that patent under 35 U.S.C. § 271(e)(2)(A), including at least claim 1.

51. Upon information and belief, Almaject's ANDA Product and/or its use in accordance with the product label satisfies each and every element of at least claim 1 of the '328 patent.

52. There is a justiciable controversy between the parties hereto as to the infringement of the '328 patent.

53. Unless enjoined by this Court, upon FDA approval of Almaject's ANDA, Almaject, Inc., Alvogen, Inc., and Alvogen PB Research and Development LLC will infringe one or more claims of the '328 patent under 35 U.S.C. § 271(a), including at least claim 1, by making, using, offering to sell, selling, and/or importing Almaject's Proposed Product in or for the United States.

54. Unless enjoined by this Court, upon FDA approval of Almaject's ANDA, Almaject, Inc., Alvogen, Inc., and Alvogen PB Research and Development LLC will induce infringement of one or more claims of the '328 patent under 35 U.S.C. § 271(b), including at least claim 1, by making, using, offering to sell, selling, and/or importing Almaject's Proposed Product in or for the United States. On information and belief, upon FDA approval of Almaject's ANDA, Almaject, Inc., Alvogen, Inc., and Alvogen PB Research and Development LLC will intentionally encourage acts of direct infringement with knowledge of the '328 patent and knowledge that their acts are encouraging infringement.

55. Unless enjoined by this Court, upon FDA approval of Almaject's ANDA, Almaject, Inc., Alvogen, Inc., and Alvogen PB Research and Development LLC will contributorily infringe one or more claims of the '328 patent under 35 U.S.C. § 271(c), including

at least claim 1, by making, using, offering to sell, selling, and/or importing Almaject's Proposed Product in or for the United States. On information and belief, Almaject, Inc., Alvogen, Inc., and Alvogen PB Research and Development LLC have had and continue to have knowledge that Almaject's Proposed Product is especially adapted for a use that infringes one or more claims of the '328 patent and that there is no substantial non-infringing use for Almaject's Proposed Product.

56. Jazz Pharmaceuticals will be substantially and irreparably damaged and harmed if Almaject, Inc., Alvogen, Inc., and Alvogen PB Research and Development LLC's infringement of the '328 patent is not enjoined.

57. Jazz Pharmaceuticals does not have an adequate remedy at law.

58. This case is an exceptional one, and Jazz Pharmaceuticals is entitled to an award of its reasonable attorneys' fees under 35 U.S.C. § 285.

Count III for Infringement of the '348 Patent

59. Jazz Pharmaceuticals repeats and realleges the allegations of the preceding paragraphs as if fully set forth herein.

60. Almaject's submission of Almaject's ANDA to obtain approval to engage in the commercial manufacture, use, sale, or offer for sale in, or importation into, the United States of Almaject's Proposed Product, prior to the expiration of the '348 patent, constitutes infringement of one or more of the claims of that patent under 35 U.S.C. § 271(e)(2)(A), including at least claim 1.

61. Upon information and belief, Almaject's ANDA Product satisfies each and every element of at least claim 1 of the '348 patent.

62. There is a justiciable controversy between the parties hereto as to the infringement of the ’348 patent.

63. Unless enjoined by this Court, upon FDA approval of Almaject’s ANDA, Almaject, Inc., Alvogen, Inc., and Alvogen PB Research and Development LLC will infringe one or more claims of the ’348 patent under 35 U.S.C. § 271(a), including at least claim 1, by making, using, offering to sell, selling, and/or importing Almaject’s Proposed Product in or for the United States.

64. Unless enjoined by this Court, upon FDA approval of Almaject’s ANDA, Almaject, Inc., Alvogen, Inc., and Alvogen PB Research and Development LLC will induce infringement of one or more claims of the ’348 patent under 35 U.S.C. § 271(b), including at least claim 1, by making, using, offering to sell, selling, and/or importing Almaject’s Proposed Product in or for the United States. On information and belief, upon FDA approval of Almaject’s ANDA, Almaject, Inc., Alvogen, Inc., and Alvogen PB Research and Development LLC will intentionally encourage acts of direct infringement with knowledge of the ’348 patent and knowledge that their acts are encouraging infringement.

65. Unless enjoined by this Court, upon FDA approval of Almaject’s ANDA, Almaject, Inc., Alvogen, Inc., and Alvogen PB Research and Development LLC will contributorily infringe one or more claims of the ’348 patent under 35 U.S.C. § 271(c), including at least claim 1, by making, using, offering to sell, selling, and/or importing Almaject’s Proposed Product in or for the United States. On information and belief, Almaject, Inc., Alvogen, Inc., and Alvogen PB Research and Development LLC have had and continue to have knowledge that Almaject’s Proposed Product is especially adapted for a use that infringes one or more claims of

the ’348 patent and that there is no substantial non-infringing use for Almaject’s Proposed Product.

66. Jazz Pharmaceuticals will be substantially and irreparably damaged and harmed if Almaject, Inc., Alvogen, Inc., and Alvogen PB Research and Development LLC’s infringement of the ’348 patent is not enjoined.

67. Jazz Pharmaceuticals does not have an adequate remedy at law.

68. This case is an exceptional one, and Jazz Pharmaceuticals is entitled to an award of its reasonable attorneys’ fees under 35 U.S.C. § 285.

PRAYER FOR RELIEF

WHEREFORE, Plaintiff Jazz Pharmaceuticals respectfully requests the following relief:

(A) A Judgment that Almaject, Inc., Alvogen, Inc., and Alvogen PB Research and Development LLC have infringed the patents-in-suit by submitting ANDA No. 216293;

(B) A Judgment that Almaject, Inc., Alvogen, Inc., and Alvogen PB Research and Development LLC have infringed, and that Almaject, Inc., Alvogen, Inc., and Alvogen PB Research and Development LLC’s making, using, selling, offering to sell, or importing Almajects’s Proposed Product will infringe, one or more claims of the patents-in-suit;

(C) An Order, pursuant to 35 U.S.C. § 271(e)(4)(A), that the effective date of FDA approval of ANDA No. 216293 be a date which is not earlier than the later of the expiration of the patents-in-suit, or any later expiration of exclusivity to which Jazz Pharmaceuticals is or becomes entitled;

(D) Preliminary and permanent injunctions enjoining Almaject, Inc., Alvogen, Inc., and Alvogen PB Research and Development LLC and their officers, agents, attorneys, and employees, and those acting in privity and/or concert with them, from making, using, offering to

sell, selling, or importing Almaject's Proposed Product until after the expiration of the patents-in-suit, or any later expiration of exclusivity to which Jazz Pharmaceuticals is or becomes entitled;

(E) A permanent injunction, pursuant to 35 U.S.C. § 271(e)(4)(B), restraining and enjoining Almaject, Inc., Alvogen, Inc., and Alvogen PB Research and Development LLC, their officers, agents, attorneys, and employees, and those acting in privity and/or concert with them, from practicing any formulations or methods claimed in the patents-in-suit, or from actively inducing or contributing to the infringement of any claim of the patents-in-suit, until after the expiration of the patents-in-suit, or any later expiration of exclusivity to which Jazz Pharmaceuticals is or becomes entitled;

(F) A Judgment that the manufacture, use, offer for sale, or sale in, and/or importation into, the United States of Almaject's Proposed Product will directly infringe, induce and/or contribute to infringement of the patents-in-suit;

(G) To the extent that Almaject, Inc., Alvogen, Inc., and Alvogen PB Research and Development LLC, their officers, agents, attorneys, and/or employees, or those acting in privity and/or concert with them, have committed any acts with respect to the formulations and methods claimed in the patents-in-suit a Judgment awarding Jazz Pharmaceuticals damages for such acts;

(H) If Almaject, Inc., Alvogen, Inc., and Alvogen PB Research and Development LLC, their officers, agents, attorneys, and/or employees, or those acting in privity and/or concert with them, engages in the manufacture, use, offer for sale, or sale in, and/or importation into, the United States of Almaject's Proposed Product prior to the expiration of the patents-in-suit, a Judgment awarding damages to Jazz Pharmaceuticals resulting from such infringement, together with interest;

(I) A Judgment declaring that the patents-in-suit remain valid and enforceable;

(J) A Judgment that this is an exceptional case pursuant to 35 U.S.C. § 285 and awarding Jazz Pharmaceuticals its attorneys' fees incurred in this action;

(K) A Judgment awarding Jazz Pharmaceuticals its costs and expenses incurred in this action; and

(L) Such further and other relief as this Court may deem just and proper.

Dated: April 16, 2025

By: s/ Charles M. Lizza

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CERTIFICATION PURSUANT TO L. CIV. R. 11.2 & 40.1

Pursuant to Local Civil Rules 11.2 and 40.1, I hereby certify that, to the best of my knowledge, the matter in controversy is not the subject of any other action pending in any court, or of any other pending arbitration or administrative proceeding.

Dated: April 16, 2025

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EXHIBIT A

US011085043B2

(12) **United States Patent**
Ignoni et al.

(10) **Patent No.: US 11,085,043 B2**
(45) **Date of Patent: Aug. 10, 2021**

(54) **EUGLOBULIN-BASED METHOD FOR DETERMINING THE BIOLOGICAL ACTIVITY OF DEFIBROTIDE**

(71) Applicant: **GENTIUM S.R.L.**, Villa Guardia (IT)

(72) Inventors: **Terenzio Ignoni**, Solbiate (IT); **Vijay Kumar**, Casnate (IT); **Khalid Islam**, Massagno (CH)

(73) Assignee: **GENTIUM S.R.L.**, Villa Guardia (IT)

(*) Notice: Subject to any disclaimer, the term of this patent is extended or adjusted under 35 U.S.C. 154(b) by 0 days.

(21) Appl. No.: **16/816,741**

(22) Filed: **Mar. 12, 2020**

(65) **Prior Publication Data**

US 2020/0208148 A1 Jul. 2, 2020

Related U.S. Application Data

(63) Continuation of application No. 15/844,801, filed on Dec. 18, 2017, now abandoned, which is a continuation of application No. 14/408,272, filed as application No. PCT/IT2012/000193 on Jun. 22, 2012, now Pat. No. 9,902,952.

(51) **Int. Cl.**

C12N 15/11	(2006.01)
C12N 9/68	(2006.01)
A61K 31/711	(2006.01)
A61K 38/36	(2006.01)
C12Q 1/37	(2006.01)
C12Q 1/56	(2006.01)
G01N 33/86	(2006.01)

(52) **U.S. Cl.**

CPC ***C12N 15/11*** (2013.01); ***A61K 31/711*** (2013.01); ***C12Q 1/37*** (2013.01); ***C12Q 1/56*** (2013.01); ***G01N 33/86*** (2013.01); *C12N 2310/127* (2013.01)

(58) **Field of Classification Search**

None

See application file for complete search history.

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(57) **ABSTRACT**

It is disclosed a method for determining the biological activity of defibrotide, which comprises the steps of: a) bringing into contact defibrotide, mammalian euglobulin and a substrate specific for the plasmin which, by reaction with the plasmin, provides a measurable product; and b) measuring the amount of product formed at successive times, to thereby determine the biological activity of the defibrotide. Liquid defibrotide formulations are also disclosed, preferably water solutions, having a defined biological activity and, in particular, having an activity of 25 to 35 IU/mg of defibrotide, preferably from 27 to 32 IU/mg and, more preferably, from 28 to 32 IU/mg.

19 Claims, 2 Drawing Sheets

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Figure 1

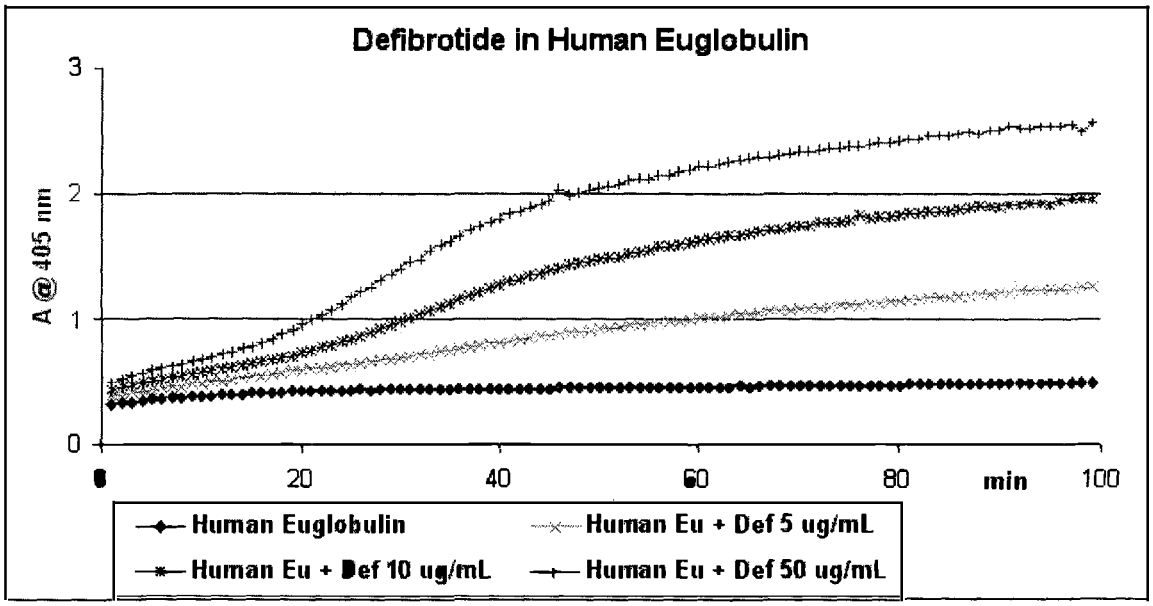


Figure 2

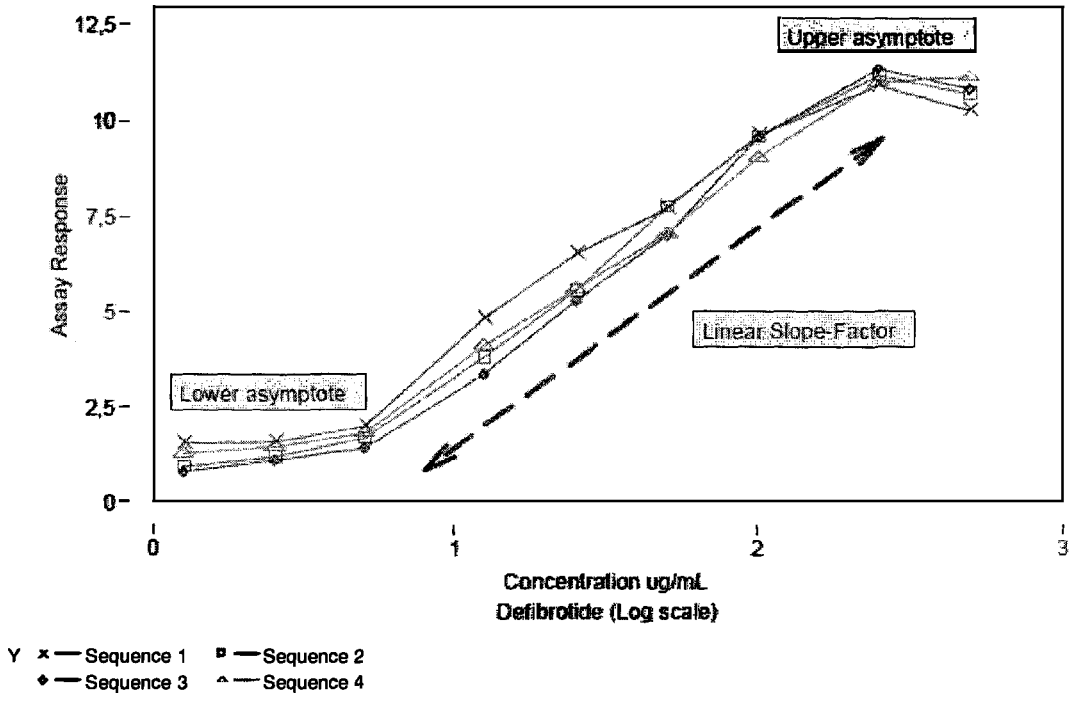
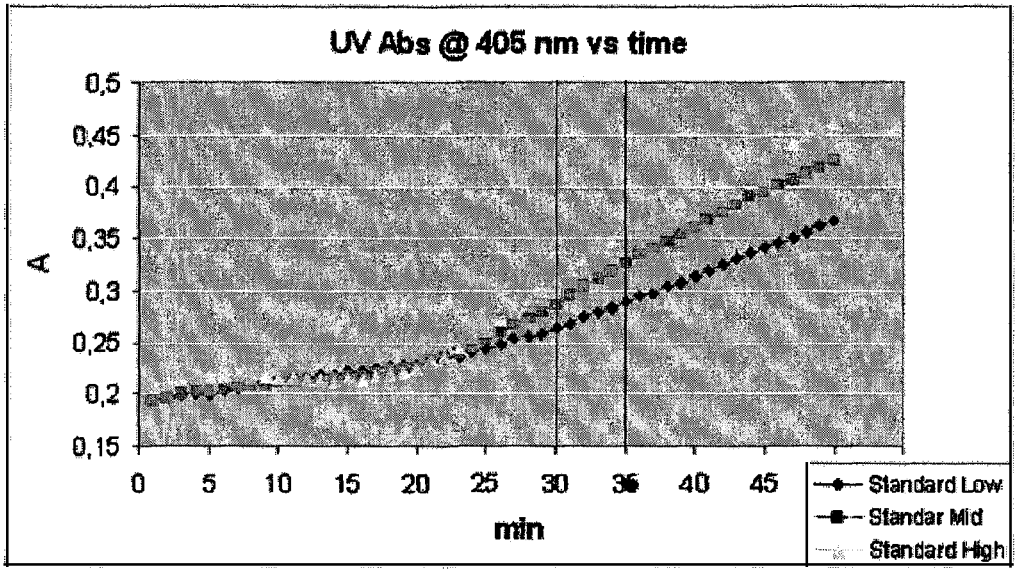


Figure 3


UV Abs @ 405 nm vs time
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EUGLOBULIN-BASED METHOD FOR DETERMINING THE BIOLOGICAL ACTIVITY OF DEFIBROTIDE

This application is a continuation of U.S. application Ser. No. 15/844,801 filed Dec. 18, 2017, which is a continuation of U.S. application Ser. No. 14/408,272 filed Dec. 15, 2014, now U.S. Pat. No. 9,902,952, and which is a 371 of international PCT/IT2012/000193, filed Jun. 22, 2012, each of which is herein incorporated by reference in its entirety.

The present invention relates to a method for determining the biological activity of defibrotide and, more especially, relates to an indirect enzymatic method for determining the biological activity of defibrotide.

TECHNICAL FIELD OF THE INVENTION

Defibrotide (Merck Index, 1996, no. 2915) is a substance of natural origin which is obtained by extraction from animal organs and which is constituted by the sodium salt of polydeoxyribonucleotides having a low molecular weight. Defibrotide has been the subject of numerous pharmacological investigations which have suggested that it be applied in therapy as an anti-thrombotic agent (U.S. Pat. No. 3,829,567).

In addition, defibrotide has also been used successfully in the treatment of peripheral arteriopathies, in acute renal insufficiency (U.S. Pat. No. 4,694,134) or in acute myocardial ischaemia (U.S. Pat. No. 4,693,995).

Defibrotide is currently undergoing clinical trials to be used for the treatment and prevention of venous occlusive disease (VOD).

Like other biological substances which are obtained by extraction, defibrotide is also subject to a limited variability of composition which is typical of natural biopolymers. A classical example of this situation is provided by heparin whose variability from batch to batch in terms of chain length, molecular weight, composition, degree of sulphatation, etc. is well known. The consequence of this is that the same amounts by weight of defibrotide could in fact be non-equivalent from the point of view of a specific biological activity.

The process of extraction, isolation and purification cannot per se ensure absolute reproducibility of the product, precisely owing to its intrinsic biopolymeric nature.

However, if well controlled, it is possible to reduce this variability: for that purpose, studies have been made of standardized industrial processes for isolating defibrotide by extraction from organs, such as, for example, that described in U.S. Pat. No. 4,985,552.

The product obtained according to the above-mentioned process is characterized by the determination of some specific physico-chemical parameters, such as, for example, electrophoretic mobility, the coefficient of extinction, optical rotatory power and mass-average relative molecular mass. However, those parameters depend basically on the structure of defibrotide and are not capable of providing information on the biological activity thereof.

As far as we know, the only methods that have been reported to be used hitherto to evaluate the biological activity of defibrotide are the fibrin plate test and the thromboelastographic recording of the euglobulin lysis time [Prino G., Mantovani M., Niada R., Coccheri S., Butti A., Indagini preliminari sull'attivita fibrinolitica, nell'animale e nell'uomo, di una nuova sostanza presente in diversi organi animali, Simposio Internazionale: La ricerca scientifica nell'industria farmaceutica in Italia, Rome, 2-4 Oct. 1975-11

Farmaco, Ed. Prat.) (1969), 24, 552-561] and the method based on plasmin disclosed in U.S. Pat. No. 7,338,777, herein incorporated by reference.

However, the above-mentioned method thromboelastographic recording of the euglobulin lysis time is characterized by considerable experimental complexity, by unsatisfactory reproducibility and precision and, in the specific case of thromboelastographic recording, by a response linearity limited to very restricted concentration ranges.

As to the plasmin-based method, the enzymatic activity of plasmin is normally determined by various standard in vitro tests. One of the most commonly used methods is the determination by spectrophotometry or fluorimetry of the chromogenic or fluorogenic compounds that are freed by the action of plasmin on suitable substrates [Haemostasis, (1978), 7, 138-145]. Peptide substrates having the formula A_1-A_2-A_3-X are generally used in which A_1 and A_2 are amino acids that are predominantly non-polar, A_3 is lysine or arginine and X represents the measurable freed compound, for example para-nitroaniline (pNa) or 2-naphthylamine (NA) [Haemostasis, (1978), 7, 146-149]. In addition to the above-mentioned peptide substrates, success has been achieved using other, simpler, compounds, such as, for example, p-nitrobenzyl-p-toluenesulphonyl-L-arginine [Haemostasis, (1978), 7, 105-108].

The rate at which the compound X is released into the incubation medium is proportional to the activity (Units/mg) of plasmin present in the sample. The method disclosed in U.S. Pat. No. 7,338,777 is thus based on the finding that, in the plasmin-evaluation tests described above, defibrotide increases the rate of release of compound X proportionally to its concentration.

However, such a method is conducted in TRIS buffer without any other plasma/serum activator/inhibitor. Therefore, the procedure does not reflect the physiological condition nor accurately simulates the mechanism of action of defibrotide in vivo.

Hitherto, therefore, no truly valid, precise and reproducible methods have been described and validated for determining the biological activity of defibrotide reflecting in an accurate way the mechanism of action of the product in a complex biological system (in vivo).

We have developed a simple and reliable method for determining the biological activity of defibrotide, which enables the samples obtained by extraction to be controlled and therefore enables medicinal preparations based on defibrotide to be standardized.

The method to which the present invention relates enables the specific biological activity of defibrotide to be determined in comparison with a reference standard with a high degree of precision and accuracy.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. **1** is a plot showing the kinetics of release of pNA from the substrate S-2251, by means of mammalian euglobulin fraction which is activated and non-activated with defibrotide (concentration 0-50 μg/ml, 0-100 min).

FIG. **2** is a plot illustrating the sigmoid that arises in relation to a standard and test sample of defibrotide.

FIG. **3** is a plot showing the "absorbance versus time" of the standard preparations (ex.: S1_Ca, S1_Cb, S1_Cc) identify a suitable linear range (ex.: from 30 to 35 min).

DESCRIPTION OF THE INVENTION

The present invention therefore relates to a method for determining the specific biological activity of samples of defibrotide, which method comprises the steps of:

a) bringing into contact defibrotide, euglobulin and a substrate specific for the plasmin which, by reaction with the plasmin, provides a measurable product and

b) measuring the amount of product formed at successive times.

The method of the invention is an indirect in vitro method for determining the activity of defibrotide, which is based on the functional interactions between defibrotide and euglobulin.

Euglobulin is that fraction of serum globulin that is insoluble in distilled water but soluble in saline solutions.

Euglobulin contains fibrinogen, PAI-1, tissue plasminogen activator (tPA), plasminogen, and to a lesser extent alpha 2-antiplasmin and also factor VIII.

The present inventors have surprisingly found that defibrotide catalyzes the hydrolysis of plasminogen into plasmin. Consequently, when defibrotide is incubated with euglobulin and a substrate specific for plasmin, such as a peptide of formula A_1-A_2-A_3-X as disclosed by Haemostasis, (1978), 7, 138-149, herein incorporated by reference, the rate at which the compound X is released into the incubation medium increases proportionally to the concentration of defibrotide itself.

In other terms, defibrotide catalyzes the hydrolysis of plasminogen contained in euglobulin into plasmin; which plasmin enzymatically reacts with the substrate specific for plasmin, preferably a cromogenic substrate, to provide a measurable product.

The method of the present invention thus further comprises the steps of: c) determining the rate of release of the measurable product during the course of the enzymatic reaction of both a standard sample and a test sample of defibrotide; d) correlating, mathematically and/or graphically, the rate of release with the corresponding defibrotide concentration to obtain the biological activity of the test sample of defibrotide.

The defibrotide sample used for the determination according to the present invention is generally prepared by extraction from organs in accordance with known procedures, such as described, for example, in U.S. Pat. No. 4,985,552 which has already been mentioned and which is also herein incorporated by reference.

A batch of normal industrially manufactured defibrotide was chosen as the reference sample (standard) and was used to prepare the calibration curves in accordance with the method of the present invention.

In general, the present method provides precise and accurate measurements of defibrotide even in the presence of contaminants, such as, for example, RNA, heparin, degraded defibrotide (defibrotide from which purin or pyrimidine has been removed) or ethanol, provided they are in concentrations, generally less than 10% by weight, such as not to impair the system.

In addition to permitting the determination of defibrotide, the method also allows the determination of other biologically equivalent substances derived from defibrotide, such as, for example, deaminated defibrotide or, more simply, defibrotide denatured/degraded by combination of physico-chemical conditions.

The present method is sufficiently sensitive to detect concentrations of defibrotide lower than or equal to 2.5 μg/ml (final concentration in the determination system) and, generally, expresses good correlation up to maximum concentration values higher than or equal to 1000 μg/ml.

The euglobulin used is generally any mammalian euglobulin fraction, such as, for example, bovine, porcine, rabbit ‘or human euglobulin, with a preference for human and bovine euglobuline.

However, although euglobulin fraction is the enzymatic system of choice, the use of other equivalent enzymatic systems, such as, for example, diluted plasma and serum (up to 1:10 with buffers), artificially created or isolated combinations of plasminogen, tPA, uPA, PAI-1&2 alpha 2 antiplasmin and the like enzymatic systems which are chemically and biologically related and have similar functionality, falls within the scope of the present invention.

In the method of the present invention, the substrate for the plasmin may be understood as being any substrate specific for plasmin which, under the conditions of the method, frees a detectable hydrolysis product X.

Depending on the nature of the detectable group X, alternative systems of detection commonly known to the person skilled in the art can be adopted equally well. Spectrophotometric or fluorimetric detection systems are particularly advantageous, especially spectrophotometric systems.

The substrates generally used are ones that are specific for plasminoge-plasmin assay. It is preferable to use peptides of the formula A_1-A_2-A_3-X, in which A_1 and A_2 are amino acids that are predominantly non-polar, A_3 is lysine or arginine and X is the detectable group. Examples of those substrates are Val-Leu-Lys-pNa, Val-Phe-Lys-pNa or pyro-Glu-Phe-Lys-pNa in which the group X detectable by spectrophotometry is para-nitroaniline (pNA). Other suitable substrates, for example Val-Gly-Arg-2NA, contain 2-naphthylamine, which is measurable by fluorimetry. A particularly preferred substrate is the compound H-D-Valyl-L-Leucyl-L-Lysine-p-nitroaniline (H-D-Val-Leu-Lys-pNA).

The specific substrates used for determining defibrotide activity in euglobulin fraction are generally commercially available.

The determination method of the present invention is carried out by placing defibrotide sample in euglobulin solution, at a specific pH and molarity.

In particular, euglobulin fractions obtained from mammalian plasma are reconstituted dissolving and diluting the euglobulin to the original volume of the generating plasma with saline buffer (ex.: the quantity of euglobulin fraction obtained from 10 mL plasma are dissolved and reconstituted to 10 mL with saline buffer at pH between 7 and 8).

However, as regards of the substrate concentrations of from 0.3 to 4 mM, preferably from 2.5 to 3.5 mM and advantageously of 3 mM, are generally used in the case of a chromogenic substrate, while concentrations of from 0.05 to 0.15 mM are used in the case of a fluorogenic substrate.

The determination method of the invention, like other enzymatic methods, is sensitive to the pH of the medium.

In fact, it cannot generally be applied at extreme pH values where the enzymatic system would be inactivated.

It is also preferable for the pH of the medium not to undergo variation at any time during the period when measurements are being taken, and therefore euglobuline fraction is reconstituted with buffer systems selected from those normally used for biological tests. Suitable buffer systems may be, for example, phosphate buffer, citrate buffer or tris(hydroxymethyl)aminomethane hydrochloride and sodium chloride (TRIS-NaCl) buffer. The reconstitution of the euglobuline fraction is preferably carried out with TRIS-NaCl.

In the present method it is usually preferred to maintain the pH of the medium in a range of approximately from 7 to 8, more preferably at approximately 7.4-7.6.

In addition, it is preferred to maintain the concentration of the buffer system in a range of from 10 to 200 mM, preferably at approximately 50 mM. More specifically for the TRIS-NaCl the concentrations should be 50 mM for TRIS and 150 mM for sodium chloride

The method of the invention for determining defibrotide biological activity, defibrotide sample solutions is diluted directly into euglobulin fraction, then the chromogenic or fluorogenic substrate is added. In particular, in order to enable the measurements it is preferable to preliminary dilute/dissolve defibrotide in TRIS-NaCl buffer in order to obtain a mother stock solution of both, sample and standard. From the mother stock solutions the sample and the standard are diluted, by serial dilution, into defined volume of euglobulin fraction till the analytical concentration range which is about 1 to 1000 μg/mL of defibrotide

An important parameter in the present method of determination is the temperature. It is preferable for the same temperature to be maintained throughout the entire duration of the measurements and for all of the samples determined, both as regards the construction of the reference curves and during the measuring stage. To that end, it is preferable to use temperature controlled apparatus and also, where necessary, it is possible to proceed with several sets of measurements, changing the position of the samples appropriately in order to ensure that the system has maximum thermal homogeneity.

Generally, this method of determination is applied in a temperature range of, for example, from 25 to 40° C., preferably from 35 to 39° C., and even more preferably at 37° C.

According to the present invention, measurement of the concentration of compound X released in the medium by the action of defibrotide starts when all of the reagents have been added and continues for a predetermined time and at a predetermined frequency as a function of the chemical nature of X and of the detection system.

Similarly to other methods of biological determination, the method of the invention also provides for a calibration stage and a measuring stage which are preferably carried out in the same microplateplate in order to reduce as far as possible the incidence of experimental variability.

The calibration stage comprises the acquisition of the absorbance data relating to the samples at known increasing concentrations of defibrotide (standard), the statistical reprocessing of those data and the extrapolation of calibration curves, which express the correlation between the increase in the rate of the enzymatic reaction of the invention and the concentration of defibrotide present in the euglobulin fraction. In the measuring stage, owing to the correlation obtained in the calibration stage, it is possible to determine the unknown biological activities of samples of defibrotide on the basis of the absorbance values measured and processed under the same conditions.

In more detail, the experimental protocol generally provides for the preparation of several samples, both standard and unknown, at various known concentrations of defibrotide. The defibrotide samples are prepared by progressive dilution of the mother solutions in accordance with a predetermined dilution factor.

In the present method, it is preferred to prepare at least 5 concentrations of the standard and 5 concentrations of the sample to be tested, preparing at least 4 replicates for each concentration of the standard and, similarly, for each concentration of the test sample, generally for successive 1:2 dilutions of mother solutions.

Both the standard and test-sample concentrations of defibrotide are generally from 0.1 to 1000 μg/ml.

The concentrations of the test sample are preferably of the same order of magnitude as the concentrations of the standard.

In accordance with the above illustration, the measurements for each concentration are preferably carried out on 1 microplates where the position of each sample, the standard and the test sample, respectively, at corresponding concentration is preferably alternated. According to this scheme for the arrangement of the samples, which is explained in more detail in the experimental part, for each concentration of both standard and test-sample defibrotide, at least 4 absorbance values are measured for each time.

The set of measurements described above are taken at predetermined times, that is to say, first of all at time to, that is to say, when all of the components have been added, before the enzymatic reaction of the invention has started, and subsequently at precise intervals and for a period of time sufficient to acquire the necessary data.

Preferably, the absorbance measurements are continued up to a maximum of 90 minutes, with readings taken every 1-10 minutes. More advantageously, the readings are taken every minute. The photometric absorbance readings are performed at a wavelength which depends on the nature of the detectable group X freed in the course of the enzymatic hydrolytic reaction. In the specific case in which X is p-NA, the absorbance is measured at 405 nm.

The absorbance readings of the standard and unknown defibrotide samples, known as raw data, generally originate directly from the same apparatus that provides for the reading operation; they are tabulated in such a manner that an absorbance value is expressed for each time and well.

The raw data are then processed, using, for example, the Spread Sheet-Microsoft Excel®. This first processing operation leads to the calculation of the average absorbance and of the associated standard deviation, at each time and for each set of readings, each set comprising at least 4 replicates for each concentration of both standard and test-sample defibrotide.

Further statistical processing of the data is carried out with commercially available software for biological assay determination as described by Finney D J, Statistical Method in Biological Assay, 2nd ed. Ch. Griffin, London and relevant Pharmacopoeias.

To be more precise, according to the present invention, defibrotide biological assay determination can be performed using parallel line model, slope ration model and four-parameter logistic curve models as defined, for example, by the relevant European Pharmacopoeia General text 5.3, Statistical Analysis"

As illustrated in FIG. **1**, by placing the time on the abscissa and the absorbance on the ordinate, straight lines will be obtained whose slope "b" will be proportional to the rate of enzymatic reaction: by increasing the concentration of defibrotide, the rate of hydrolysis and, proportionally, the value of "b" will increase. Finally, the slope values, calculated as described above for each set of replicates of standard defibrotide and test-sample defibrotide, are correlated with the concentration of defibrotide to which they relate. Suitable mathematical transformation of the abscissa (i.e. log of defibrotide concentration) can be used in lieu of the real value.

Graphically, that correlation gives rise to a sigmoid for the standard and a sigmoid for the test sample (FIG. **2**); the

central portions of the sigmoid have two straight lines which are generally parallel and the distance between which is a function of the difference in biological activity between the test sample and the standard. This interval is used for potency determination using the parallel line model as described by Finney D J, Statistical Method in Biological Assay, 2nd ed. Ch. Griffin, London.

For a more specific determination, the four-parameter logistic curve models is used and in this case the entire sigmoid curve of both, sample and standard, is used for the calculation of the relative biological potency of the sample.

In a preferred embodiment of the present invention, the standard solutions and the solutions of the samples of defibrotide to be determined are introduced into the respective wells of the microplate. The euglobulin fraction are prepared at the moment of use and it is the dilution media of defibrotide stock solution. Finally, the solution containing the chromogenic substrate is added. The microplate is then placed in the thermostated reader and, after rapid agitation, readings of the system's absorbance are taken at predetermined intervals and for the predetermined period of time. The raw data obtained are then processed, thus determining the unknown activities of the defibrotide samples.

As it shall be appreciated from the following examples, the method according to the present invention allows to obtain liquid defibrotide formulations, preferably water solutions, having a defined biological activity and, in particular, having an activity of 25 to 35 IU/mg of defibrotide, preferably from 27.5 to 32.5 IU/mg and, more preferably, from 28 to 32 IU/mg.

Liquid defibrotide formulations are preferably marketed in form of containers, more preferably vials, containing 200 mg of defibrotide in 2.5 ml of buffered water solution (preferably at a pH from 6.5 to 8.5, more preferably from 7 to 8), to be diluted before use; consequently, when the biological activity is assessed with the method of the present invention, each container presents a defibrotide activity of 5000 to 7000 IU, preferably 5500 to 6500 IU, more preferably 5600 to 6400.

Those and other aspects of the invention will be better illustrated in the following examples which are not, however, to be regarded as limiting the invention.

EXAMPLES

The following materials were used in the examples given here:

Apparatus

Main features:	Microplate reader for UV-Vis absorbance determination equipped with thermostatic chamber and absorbance filter at 405 nm.		
Detection	Kinetic Absorbance determination at 405 nm		
Plate Type	96-well clear for UV-Vis Determination		
Chamber Temperature	37° C.	Total Absorbance recording time	45-60 min
Absorbance recording frequency	About 1 x min		

Programs & Software

Microsoft Excel® (Microsoft Corporation, Redmond, Wash., USA) Substances

Defibrotide (Gentium)

Chromogenic substrate S-2251 (Chromogenix Instrumentation Laboratory S.p.A., Milan, Italy)

Tris(hydroxymethyl)aminomethane (TRIS)-NaCl, (Sigma-Aldrich, Milan, Italy)

1N HCl (Carlo Erba reagenti, Milan, Italy)

IN NaOH (Carlo Erba reagenti, Milan, Italy)

Bovine Plasma (Tebu Bio Italia, Magenta (MI), Italy)

Glacial Acetic Acid (Carlo Erba reagenti, Milan, Italy)

Solutions

TRIS-NaCl (1 L Preparation): Into a 1 L beaker quantitatively transfer 6.06 g of TRIS-HCl and 2.2 g of NaCl. Dissolve in 500 mL of purified water and adjust the pH to 7.4 with about 40 mL of HCl 1N. Quantitatively transfer the solution into a volumetric flask of 1 L and dilute the solution to volume with purified water. Store the solution into refrigerator (2-8° C.)

Chromogenic Substrate S2251 (CAS 63589-93-5) 3 mM (15.2 mL Preparation): Dissolve about 25 mg of chromogenic substrate with 15.2 mL of purified water. Store the solution into refrigerator (2-8° C.)

Bovine euglobulins (10 mL Preparation). In a container with a minimum capacity of 300 mL introduce 240 mL of ice-cooled purified water and under stirring add 10 mL bovine plasma. Adjust the pH to 5.3±0.1 with acetic acid 1%. Allow to settle at 2-8° C. for 1 to 16 hours. Remove the clear supernatant solution by siphoning and collect the precipitate by centrifugation at 2.800 rpm for 5 minutes and 4° C. Suspend the precipitate dispersing mechanically (e.g.: by means of a laboratory glass rod) in 5 mL of ice-cooled purified water, shake for about 5 min and collect the precipitate by centrifugation at 2.800 rpm for 5 minutes and 4° C. Disperse the precipitate mechanically into about 10 mL of TRIS-NaCl; to facilitate the dissolution of the precipitate crush the particles of the precipitate with a suitable instrument (es.: laboratory glass rod). Store the obtained suspension at 2-8° C. for not less than 1 hour and not more than 6 hours before its use.

Standard and Sample Defibrotide Solutions

Reference Stock solution

Into 20 mL volumetric flask quantitatively transfer about 100 mg of defibrotide reference standard accurately weighed. Dissolve the powder with about 10 mL TRIS-NaCl and bring the volume with the same solvent. Dilute 1:4 the obtained solution with TRIS-NaCl in order to obtain a defibrotide RS solution of about 1.25 mg/mL.

Sample Stock Solution

Into 20 mL volumetric flask quantitatively transfer about 100 mg of defibrotide sample accurately weighed. Dissolve the powder with about 10 mL TRIS-NaCl and bring the volume with the same solvent. Dilute 1:4 the obtained solution with TRIS-NaCl in order to obtain a defibrotide sample solution of about 1.25 mg/mL.

Reference and Sample Solutions Preparation

a) Defibrotide 125 ug/mL: dilute 1:10 defibrotide stock solution (Reference and Sample) with TRIS NaCl (corresponding to 50 ug/mL into the plate well). Into an eppendorf tube quantitatively transfer 500 uL of the prepared solution and mix with 500 uL of euglobulin. Close the tube and store in ice-cooled water.

b) Defibrotide 62.5 ug/mL: dilute 1:2 solution (a) with TRIS NaCl (corresponding to 25 ug/mL into the plate well), Into an eppendorf tube quantitatively transfer 500 uL of the prepared solution and mix with 500 uL of euglobulin. Close the tube and store in ice-cooled water.

c) Defibrotide 31.25 ug/mL: dilute 1:2 solution (b) with TRIS NaCl (corresponding to 12.5 ug/mL into the plate well). Into an eppendorf tube quantitatively transfer 500 uL of the prepared solution and mix with 500 uL of euglobulin. Close the tube and store in ice-cooled water.

d) Defibrotide 12.5 ug/mL: dilute 1:2.5 solution (d) with TRIS NaCl (corresponding to 5 ug/mL into the plate well). Into an eppendorf tube quantitatively transfer 500 uL of the prepared solution and mix with 500 uL of euglobulin. Close the tube and store in ice-cooled water.

Blank Solution

Mix 1 volume of euglobulins with 1 volume of TRIS NaCl solution (ex.: 500 uL+500 uL)

Plate Deposition

According to the proposed deposition scheme (see Table 1 below) add in each well of the plate 200 uL of standard or sample or blank solution. Note different deposition scheme can be used according to the availability and configuration of automatic pipettes. However not less than 4 depositions for each reference and sample solution must be used for the assay.

Immediately before incubation of the plate into the microplate reader add in each well 50 uL of Chromogenic substrate.

TABLE 1

	1	2	3	4	5	6	7	8	9	10	11	12
A	—	BLK	S1__Ca	S1__Cb	S1__Cc	S1__Cd	—	—	—	—	—	—
B	—	BLK	S2__Ca	S2__Cb	S2__Cc	S2__Cd	—	—	—	—	—	—
C	—	BLK	S3__Ca	S3__Cb	S3__Cc	S3__Cd	—	—	—	—	—	—
D	—	BLK	S4__Ca	S4__Cb	S4__Cc	S4__Cd	—	—	—	—	—	—
E	—	BLK	U1__Ca	U1__Cb	U1__Cc	U1__Cd	—	—	—	—	—	—
F	—	BLK	U2__Ca	U2__Cb	U2__Cc	U2__Cd	—	—	—	—	—	—
G	—	BLK	U3__Ca	U3__Cb	U3__Cc	U3__Cd	—	—	—	—	—	—
H	—	BLK	U4__Ca	U4__Cb	U4__Cc	U4__Cd	—	—	—	—	—	—

S1, S2, S3, S4: Reference Solution deposition 1, deposition 2, deposition 3, deposition 4, U1, U2, U2, U5: Sample solution deposition 1, deposition 2 deposition 3, deposition 4, Ca, Cb, Cc etc.: Defibrotide reference and sample concentration a, b, c etc.
BLK Blank solution

Calculation and Results

From the kinetic plot "absorbance versus time" of the standard preparations (ex.: S1_Ca, S1_Cb, S1_Cc) identify a suitable linear range (ex.: from 30 to 35 min, see FIG. **3**).

Identification of the linear kinetic range (A@405 nm vs time).

Calculate for each preparation of the standard and of the sample the response of the assay (Slope) in the pre-defined time range as follow.

$$\text{Sample \& Standard Response} = \frac{A_b - A_a}{T_b - T_a} \times 1000$$

Where:

Aa is the Absorbance values at Time Ta (30 min from the plot above)

Ab is the Absorbance value at Time Tb (35 min from the plot above)

Report the obtained value in a tabular format as reported in table 2.

TABLE 2

Concentration	Standard Preparation				Sample Preparation			
Level [ug/mL]	S1	S2	S3	S4	U1	U2	U3	U4
5	S1__Cd	S2__Cd	S3__Cd	S4__Cd	U1__Cd	U2__Cd	U3__Cd	U4__Cd
12.5	S1__Cc	S2__Cc	S3__Cc	S4__Cc	U1__Cc	U2__Cc	U3__Cc	U4__Cc
25	S1__Cb	S2__Cb	S3__Cb	S4__Cb	U1__Cb	U2__Cb	U3__Cb	U4__Cb
50	S1__Ca	S2__Ca	S3__Ca	S4__Ca	U1__Ca	U2__Ca	U3__Ca	U4__Ca

Plot the responses for the substance to be examined and for the standard against the logarithms of the concentration and calculate the activity of the substance to be examined using the parallel line model as defined by the relevant 5.3.2 chapter of the Ph. Eur Current edition.

Not less than 3 consecutive serial dilutions of the reference and of the sample should be used (e.g., defibrotide concentration 5 μg/mL, 12.5 μg/mL, 25 μg/mL, 50 g/mL, or 5 μg/mL, 12.5 μg/mL, 25 μg/mL, or 12.5 μg/mL, 25 μg/mL, 50 g/mL).

Analysis of the Variance

The analysis of the variance is performed according to the section 5.3.2.3 of the Ph. Eur. current edition and Finney D J (1964) Statistical Method in Biological Assay 2nd ed.

Test for Validity

1) The linear regression terms is significant, i.e. the calculated probability is less than 0.05. If this criterion is not met, it is not possible to calculate 95% C.I.
2) The Term of non-parallelism is not significant, i.e. the calculated probability is less than 0.05.
3) The term for non linearity is not significant, i.e. the calculated probability is less than 0.05.

Acceptance Criteria

4) The estimated potency is not less than 90% and not more than 111% of the stated potency.
5) The confidence limits (P=0.95) of the estimated potency are not less than 80% and not more than 125% of the stated potency

Calculation

$$\text{Sample Potency } (UI/\text{mg}) = \frac{R \times \text{Std Potency } (UI/\text{mg}) \times \text{Conc. Std (mg/mL)}}{\text{Conc. Sample (mg/mL)}}$$

where:

R: Result of sample obtained by the parallel line model analysis

Std Potency: Stated potency of the Standard (UI/mg on dry substance)

Conc. Std: Concentration of the Standard (mg/mL on dry substance)

Conc. Sample: Concentration of the Sample (mg/mL on dry substance)

Assay Applied to Defibrotide Formulations

The above-disclosed assay has been used to determine the biological activity of liquid formulations containing 200 mg of defibrotide in 2.5 ml (80 mg per ml) and having the quali-quantitative composition reported in table 3.

TABLE 3

Component	Reference to Standard Quality	Function	200 mg Injection	Concentration per mL
Defibrotide	In-house standard	Drug Substance	200.00 mg	80.00 mg
Sodium Citrate, Dihydrate	USP - EP	Buffer	25.00 mg	10.00 mg
Water-for-injection	USP - EP	Solvent/vehicle	q.s. to 2.5 mL	1.00 mL
Sodium hydroxide 1M or hydrochloric acid 1M	NF - EP	pH adjustment	q.s. to 6.8-7.8	—
Nitrogen	NF - EP	Inert gas to displace the air	q.s.	—

The results are reported in table 4.

TABLE 4

Batch	Potency IU/mg	Potency IU/container of 200 mg
688	31	6200
738	34	6800
785	32	6400
836	30	6000
0406	31	6200
DS0617	35	7000
DV0502*	*	Not available
DV0601	35	7000
1070010073	32	6400
1080010016	32	6400
1080020018	31	6200
1080030021	31	6200
1080040110	32	6400
1080050114	35	7000
1080060117	34	6800
Mean	33	
Min	30	
Max	35	
RSD (%)	5.4	

*Used as Reference Standard

The invention claimed is:

1. A defibrotide formulation consisting of defibrotide, sodium citrate, and water for injection, having a potency of 25 to 35 IU/mg, and a concentration of at least 80 mg/mL, wherein the defibrotide potency is determined by a method comprising the steps of:

a) bringing into contact defibrotide, mammalian euglobulin and a substrate specific for plasmin which, by reaction with plasmin, provides a measurable product; and

b) measuring the amount of product formed at successive times, to thereby determine the potency of the defibrotide.

2. The defibrotide formulation of claim **1**, wherein the formulation has a potency of 27.5 to 32.5 IU/mg.

3. The defibrotide formulation of claim **2**, wherein the formulation has a potency of 28 to 32 IU/mg.

4. The defibrotide formulation of claim **1**, wherein the formulation is a water solution.

5. The defibrotide formulation of claim **4**, wherein the formulation has a pH of from 6.5 to 8.5.

6. The defibrotide formulation of claim **5**, wherein the formulation has a pH of from 7 to 8.

7. The defibrotide formulation of claim **1**, wherein the euglobulin is human, rabbit or bovine euglobulin.

8. The defibrotide formulation of claim **1**, wherein plasmin which reacts with the substrate specific for plasmin is released by plasminogen contained in the mammalian euglobulin.

9. The liquid defibrotide formulation of claim **1**, wherein the substrate specific for the plasmin is a chromogenic substrate.

10. The defibrotide formulation of claim **1**, wherein the substrate specific for the plasmin is a compound of formula A1-A2-A3-X in which A1 and A2 are non-polar amino acids, A3 is lysine or arginine and X is the measurable product.

11. The defibrotide formulation of claim **10**, wherein the measurable product X is selected from the group consisting of para-nitroaniline and 2-naphthylamine.

12. The defibrotide formulation of claim **10**, wherein the substrate specific for plasmin is H-D-Valyl-L-Leucyl-L-Lysine-p-nitroaniline.

13. The defibrotide formulation of claim **10**, wherein the measurable product X is measured by spectrophotometry or spectrofluorimetry.

14. The defibrotide formulation of claim **1**, wherein the mammalian euglobulin is obtained from a volume of plasma and reconstituted to the same volume of the originating plasma or diluted up to 1:10 with suitable buffer and the substrate specific for the plasmin is a chromogenic/fluorogenic substrate having a concentration of from 2.5 to 3.5 mM.

15. The defibrotide formulation of claim **1**, wherein said method is carried out in a reaction medium which is an aqueous solution buffered to a pH of from 7 to 8.

16. The defibrotide formulation of claim **1**, wherein the method is maintained at a temperature of from 35 to 39° C.

17. The defibrotide formulation of claim **1**, wherein the substrate specific for plasmin has a concentration of from 0.3 to 4 mM.

18. The defibrotide formulation of claim **1**, wherein the method comprises the steps of: c) determining the rate of release of the measurable product during the course of the reaction of both a standard sample and a test sample of defibrotide; d) correlating the rate of release with the corresponding defibrotide concentration to obtain the potency of the test sample of defibrotide.

19. The defibrotide formulation of claim **17**, wherein the concentration of the substrate specific for plasmin is 3 mM.

* * * * *

EXHIBIT B

US011236328B2

(12) **United States Patent**
Ignoni et al.

(10) **Patent No.: US 11,236,328 B2**
(45) **Date of Patent: *Feb. 1, 2022**

(54) **EUGLOBULIN-BASED METHOD FOR DETERMINING THE BIOLOGICAL ACTIVITY OF DEFIBROTIDE**

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(*) Notice: Subject to any disclaimer, the term of this patent is extended or adjusted under 35 U.S.C. 154(b) by 0 days.

This patent is subject to a terminal disclaimer.

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(63) Continuation of application No. 17/396,028, filed on Aug. 6, 2021, which is a continuation of application No. 16/816,741, filed on Mar. 12, 2020, now Pat. No. 11,085,043, which is a continuation of application No. 15/844,801, filed on Dec. 18, 2017, now abandoned, which is a continuation of application No. 14/408,272, filed as application No. PCT/IT2012/000193 on Jun. 22, 2012, now Pat. No. 9,902,952.

(51) **Int. Cl.**

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A61K 38/36	(2006.01)
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(58) **Field of Classification Search**

None

See application file for complete search history.

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(57) **ABSTRACT**

It is disclosed a method for determining the biological activity of defibrotide, which comprises the steps of: a) bringing into contact defibrotide, mammalian euglobulin and a substrate specific for the plasmin which, by reaction with the plasmin, provides a measurable product; and b) measuring the amount of product formed at successive times, to thereby determine the biological activity of the defibrotide. Liquid defibrotide formulations are also disclosed, preferably water solutions, having a defined biological activity and, in particular, having an activity of 25 to 35 IU/mg of defibrotide, preferably from 27 to 32 IU/mg and, more preferably, from 28 to 32 IU/mg.

19 Claims, 2 Drawing Sheets

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Figure 1

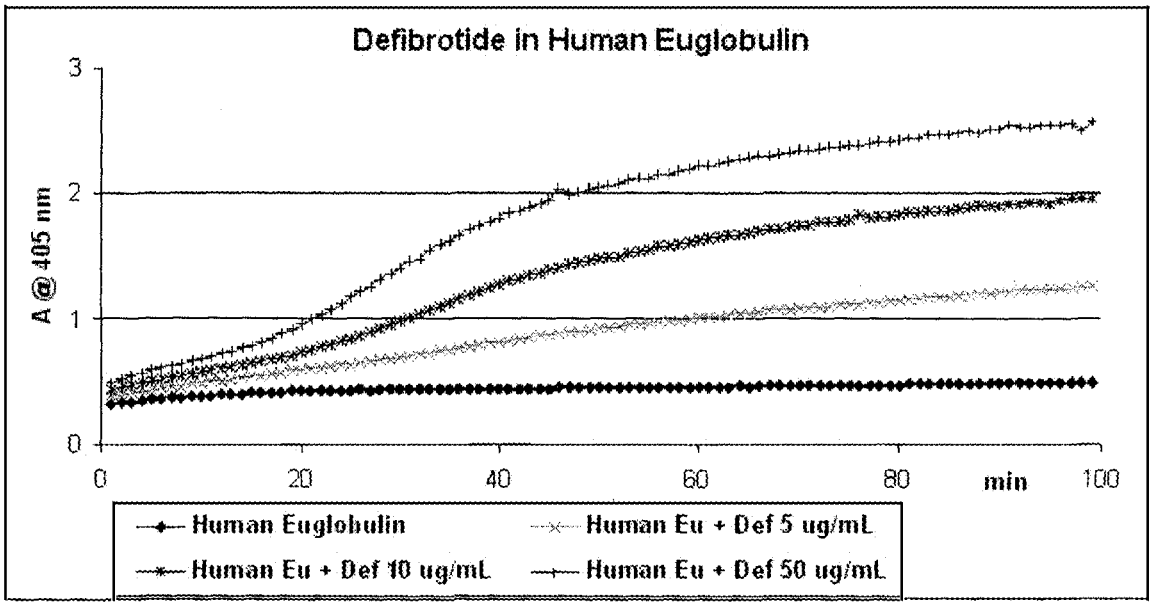


Figure 2

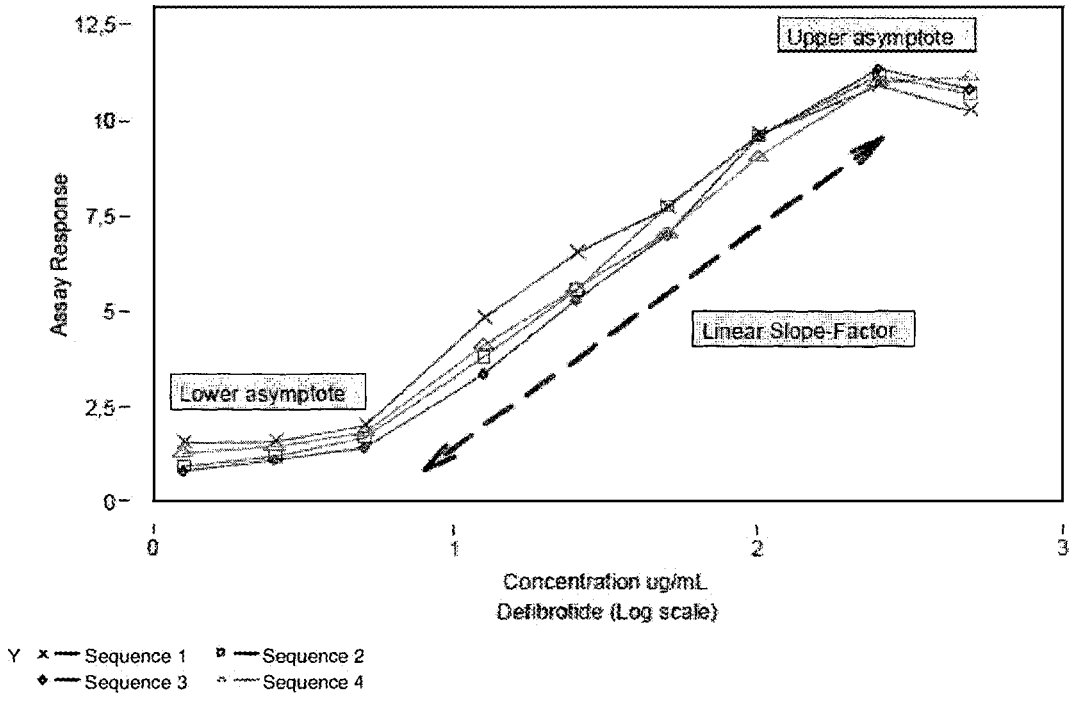
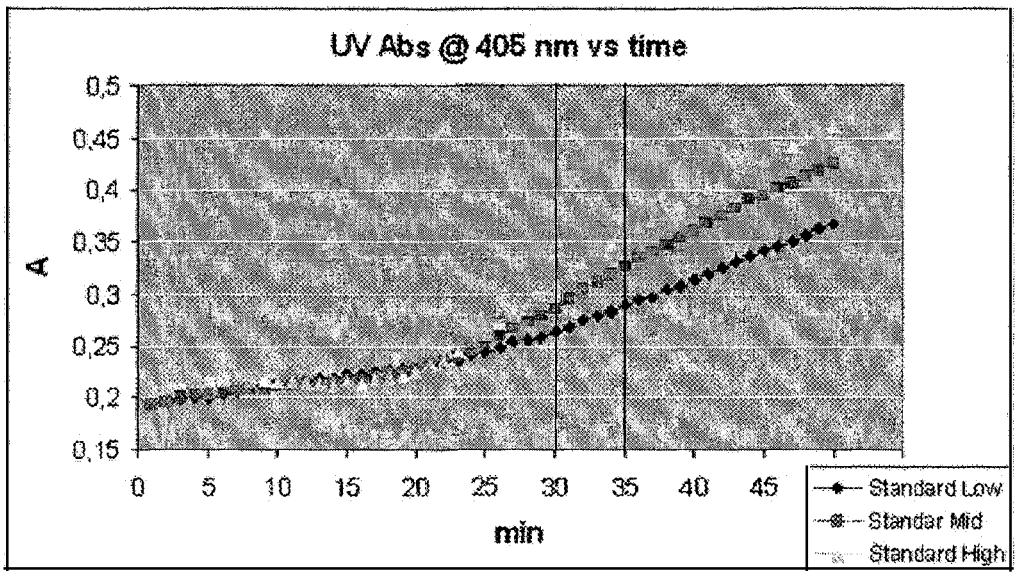


Figure 3


UV Abs @ 405 nm vs time
A
0,5
0,45
0,4
0,35
0,3
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0,2
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10
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20
25
30
35
40
45
min
Standard Low
Standar Mid
Standard High

EUGLOBULIN-BASED METHOD FOR DETERMINING THE BIOLOGICAL ACTIVITY OF DEFIBROTIDE

This application is a continuation of U.S. application Ser. No. 17/396,028, filed Aug. 6, 2021, which is a continuation of U.S. application Ser. No. 16/816,741, filed Mar. 12, 2020, now U.S. Pat. No. 11,085,043, which is a continuation of U.S. application Ser. No. 15/844,801 filed Dec. 18, 2017, now abandoned, which is a continuation of U.S. application Ser. No. 14/408,272 filed Dec. 15, 2014, now U.S. Pat. No. 9,902,952, and which is a 371 of international PCT/IT2012/000193, filed Jun. 22, 2012, each of which is herein incorporated by reference in its entirety.

The present invention relates to a method for determining the biological activity of defibrotide and, more especially, relates to an indirect enzymatic method for determining the biological activity of defibrotide.

TECHNICAL FIELD OF THE INVENTION

Defibrotide (Merck Index, 1996, no. 2915) is a substance of natural origin which is obtained by extraction from animal organs and which is constituted by the sodium salt of polydeoxyribonucleotides having a low molecular weight. Defibrotide has been the subject of numerous pharmacological investigations which have suggested that it be applied in therapy as an anti-thrombotic agent (U.S. Pat. No. 3,829,567).

In addition, defibrotide has also been used successfully in the treatment of peripheral arteriopathies, in acute renal insufficiency (U.S. Pat. No. 4,694,134) or in acute myocardial ischaemia (U.S. Pat. No. 4,693,995).

Defibrotide is currently undergoing clinical trials to be used for the treatment and prevention of venous occlusive disease (VOD).

Like other biological substances which are obtained by extraction, defibrotide is also subject to a limited variability of composition which is typical of natural biopolymers. A classical example of this situation is provided by heparin whose variability from batch to batch in terms of chain length, molecular weight, composition, degree of sulphatation, etc. is well known. The consequence of this is that the same amounts by weight of defibrotide could in fact be non-equivalent from the point of view of a specific biological activity.

The process of extraction, isolation and purification cannot per se ensure absolute reproducibility of the product, precisely owing to its intrinsic biopolymeric nature.

However, if well controlled, it is possible to reduce this variability: for that purpose, studies have been made of standardized industrial processes for isolating defibrotide by extraction from organs, such as, for example, that described in U.S. Pat. No. 4,985,552.

The product obtained according to the above-mentioned process is characterized by the determination of some specific physico-chemical parameters, such as, for example, electrophoretic mobility, the coefficient of extinction, optical rotatory power and mass-average relative molecular mass. However, those parameters depend basically on the structure of defibrotide and are not capable of providing information on the biological activity thereof.

As far as we know, the only methods that have been reported to be used hitherto to evaluate the biological activity of defibrotide are the fibrin plate test and the thromboelastographic recording of the euglobulin lysis time [Prino G., Mantovani M., Niada R., Coccheri S., Butti A., Indagini preliminari sull'attivita fibrinolitica, nell'animale e nell'uomo, di una nuova sostanza presente in diversi organi animali, Simposio Internazionale: La ricerca scientifica nell'industria farmaceutica in Italia, Rome, 2-4 Oct. 1975-11 Farmaco, Ed. Prat.) (1969), 24, 552-561] and the method based on plasmin disclosed in U.S. Pat. No. 7,338,777, herein incorporated by reference.

However, the above-mentioned method thromboelastographic recording of the euglobulin lysis time is characterized by considerable experimental complexity, by unsatisfactory reproducibility and precision and, in the specific case of thromboelastographic recording, by a response linearity limited to very restricted concentration ranges.

As to the plasmin-based method, the enzymatic activity of plasmin is normally determined by various standard in vitro tests. One of the most commonly used methods is the determination by spectrophotometry or fluorimetry of the chromogenic or fluorogenic compounds that are freed by the action of plasmin on suitable substrates [Haemostasis, (1978), 7, 138-145]. Peptide substrates having the formula A_1-A_2-A_3-X are generally used in which A_1 and A_2 are amino acids that are predominantly non-polar, A_3 is lysine or arginine and X represents the measurable freed compound, for example para-nitroaniline (pNa) or 2-naphthylamine (NA) [Haemostasis, (1978), 7, 146-149]. In addition to the above-mentioned peptide substrates, success has been achieved using other, simpler, compounds, such as, for example, p-nitrobenzyl-p-toluenesulphonyl-L-arginine [Haemostasis, (1978), 7, 105-108].

The rate at which the compound X is released into the incubation medium is proportional to the activity (Units/mg) of plasmin present in the sample. The method disclosed in U.S. Pat. No. 7,338,777 is thus based on the finding that, in the plasmin-evaluation tests described above, defibrotide increases the rate of release of compound X proportionally to its concentration.

However, such a method is conducted in TRIS buffer without any other plasma/serum activator/inhibitor. Therefore, the procedure does not reflect the physiological condition nor accurately simulates the mechanism of action of defibrotide in vivo.

Hitherto, therefore, no truly valid, precise and reproducible methods have been described and validated for determining the biological activity of defibrotide reflecting in an accurate way the mechanism of action of the product in a complex biological system (in vivo).

We have developed a simple and reliable method for determining the biological activity of defibrotide, which enables the samples obtained by extraction to be controlled and therefore enables medicinal preparations based on defibrotide to be standardized.

The method to which the present invention relates enables the specific biological activity of defibrotide to be determined in comparison with a reference standard with a high degree of precision and accuracy.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. **1** is a plot showing the kinetics of release of pNA from the substrate S-2251, by means of mammalian euglobulin fraction which is activated and non-activated with defibrotide (concentration 0-50 μg/ml, 0-100 min).

FIG. **2** is a plot illustrating the sigmoid that arises in relation to a standard and test sample of defibrotide.

FIG. **3** is a plot showing the "absorbance versus time" of the standard preparations (ex.: S1_Ca, S1_Cb, S1_Cc) identify a suitable linear range (ex.: from 30 to 35 min).

DESCRIPTION OF THE INVENTION

The present invention therefore relates to a method for determining the specific biological activity of samples of defibrotide, which method comprises the steps of:

a) bringing into contact defibrotide, euglobulin and a substrate specific for the plasmin which, by reaction with the plasmin, provides a measurable product and

b) measuring the amount of product formed at successive times.

The method of the invention is an indirect in vitro method for determining the activity of defibrotide, which is based on the functional interactions between defibrotide and euglobulin.

Euglobulin is that fraction of serum globulin that is insoluble in distilled water but soluble in saline solutions.

Euglobulin contains fibrinogen, PAI-1, tissue plasminogen activator (tPA), plasminogen, and to a lesser extent alpha 2-antiplasmin and also factor VIII.

The present inventors have surprisingly found that defibrotide catalyzes the hydrolysis of plasminogen into plasmin. Consequently, when defibrotide is incubated with euglobulin and a substrate specific for plasmin, such as a peptide of formula A_1-A_2-A_3-X as disclosed by Haemostasis, (1978), 7, 138-149, herein incorporated by reference, the rate at which the compound X is released into the incubation medium increases proportionally to the concentration of defibrotide itself.

In other terms, defibrotide catalyzes the hydrolysis of plasminogen contained in euglobulin into plasmin; which plasmin enzymatically reacts with the substrate specific for plasmin, preferably a chromogenic substrate, to provide a measurable product.

The method of the present invention thus further comprises the steps of: c) determining the rate of release of the measurable product during the course of the enzymatic reaction of both a standard sample and a test sample of defibrotide; d) correlating, mathematically and/or graphically, the rate of release with the corresponding defibrotide concentration to obtain the biological activity of the test sample of defibrotide.

The defibrotide sample used for the determination according to the present invention is generally prepared by extraction from organs in accordance with known procedures, such as described, for example, in U.S. Pat. No. 4,985,552 which has already been mentioned and which is also herein incorporated by reference.

A batch of normal industrially manufactured defibrotide was chosen as the reference sample (standard) and was used to prepare the calibration curves in accordance with the method of the present invention.

In general, the present method provides precise and accurate measurements of defibrotide even in the presence of contaminants, such as, for example, RNA, heparin, degraded defibrotide (defibrotide from which purin or pyrimidine has been removed) or ethanol, provided they are in concentrations, generally less than 10% by weight, such as not to impair the system.

In addition to permitting the determination of defibrotide, the method also allows the determination of other biologically equivalent substances derived from defibrotide, such as, for example, deaminated defibrotide or, more simply, defibrotide denatured/degraded by combination of physico-chemical conditions.

The present method is sufficiently sensitive to detect concentrations of defibrotide lower than or equal to 2.5 μg/ml (final concentration in the determination system) and, generally, expresses good correlation up to maximum concentration values higher than or equal to 1000 μg/ml.

The euglobulin used is generally any mammalian euglobulin fraction, such as, for example, bovine, porcine, rabbit or human euglobulin, with a preference for human and bovine euglobuline.

However, although euglobulin fraction is the enzymatic system of choice, the use of other equivalent enzymatic systems, such as, for example, diluted plasma and serum (up to 1:10 with buffers), artificially created or isolated combinations of plasminogen, tPA, uPA, PAI-1&2 alpha 2 antiplasmin and the like enzymatic systems which are chemically and biologically related and have similar functionality, falls within the scope of the present invention.

In the method of the present invention, the substrate for the plasmin may be understood as being any substrate specific for plasmin which, under the conditions of the method, frees a detectable hydrolysis product X.

Depending on the nature of the detectable group X, alternative systems of detection commonly known to the person skilled in the art can be adopted equally well. Spectrophotometric or fluorimetric detection systems are particularly advantageous, especially spectrophotometric systems.

The substrates generally used are ones that are specific for plasminoge-plasmin assay. It is preferable to use peptides of the formula A_1-A_2-A_3-X, in which A_1 and A_2 are amino acids that are predominantly non-polar, A_3 is lysine or arginine and X is the detectable group. Examples of those substrates are Val-Leu-Lys-pNa, Val-Phe-Lys-pNa or pyro-Glu-Phe-Lys-pNa in which the group X detectable by spectrophotometry is para-nitroaniline (pNA). Other suitable substrates, for example Val-Gly-Arg-2NA, contain 2-naphthylamine, which is measurable by fluorimetry. A particularly preferred substrate is the compound H-D-Valyl-L-Leucyl-L-Lysine-p-nitroaniline (H-D-Val-Leu-Lys-pNA).

The specific substrates used for determining defibrotide activity in euglobulin fraction are generally commercially available.

The determination method of the present invention is carried out by placing defibrotide sample in euglobulin solution, at a specific pH and molarity.

In particular, euglobulin fractions obtained from mammalian plasma are reconstituted dissolving and diluting the euglobulin to the original volume of the generating plasma with saline buffer (ex.: the quantity of euglobulin fraction obtained from 10 mL plasma are dissolved and reconstituted to 10 mL with saline buffer at pH between 7 and 8).

However, as regards of the substrate concentrations of from 0.3 to 4 mM, preferably from 2.5 to 3.5 mM and advantageously of 3 mM, are generally used in the case of a chromogenic substrate, while concentrations of from 0.05 to 0.15 mM are used in the case of a fluorogenic substrate.

The determination method of the invention, like other enzymatic methods, is sensitive to the pH of the medium.

In fact, it cannot generally be applied at extreme pH values where the enzymatic system would be inactivated.

It is also preferable for the pH of the medium not to undergo variation at any time during the period when measurements are being taken, and therefore euglobuline fraction is reconstituted with buffer systems selected from

those normally used for biological tests. Suitable buffer systems may be, for example, phosphate buffer, citrate buffer or tris(hydroxymethyl)aminomethane hydrochloride and sodium chloride (TRIS-NaCl) buffer. The reconstitution of the euglobuline fraction is preferably carried out with TRIS-NaCl.

In the present method it is usually preferred to maintain the pH of the medium in a range of approximately from 7 to 8, more preferably at approximately 7.4-7.6.

In addition, it is preferred to maintain the concentration of the buffer system in a range of from 10 to 200 mM, preferably at approximately 50 mM. More specifically for the TRIS-NaCl the concentrations should be 50 mM for TRIS and 150 mM for sodium chloride

The method of the invention for determining defibrotide biological activity, defibrotide sample solutions is diluted directly into euglobulin fraction, then the chromogenic or fluorogenic substrate is added. In particular, in order to enable the measurements it is preferable to preliminary dilute/dissolve defibrotide in TRIS-NaCl buffer in order to obtain a mother stock solution of both, sample and standard. From the mother stock solutions the sample and the standard are diluted, by serial dilution, into defined volume of euglobulin fraction till the analytical concentration range which is about 1 to 1000 μg/mL of defibrotide

An important parameter in the present method of determination is the temperature. It is preferable for the same temperature to be maintained throughout the entire duration of the measurements and for all of the samples determined, both as regards the construction of the reference curves and during the measuring stage. To that end, it is preferable to use temperature controlled apparatus and also, where necessary, it is possible to proceed with several sets of measurements, changing the position of the samples appropriately in order to ensure that the system has maximum thermal homogeneity.

Generally, this method of determination is applied in a temperature range of, for example, from 25 to 40° C., preferably from 35 to 39° C., and even more preferably at 37° C.

According to the present invention, measurement of the concentration of compound X released in the medium by the action of defibrotide starts when all of the reagents have been added and continues for a predetermined time and at a predetermined frequency as a function of the chemical nature of X and of the detection system.

Similarly to other methods of biological determination, the method of the invention also provides for a calibration stage and a measuring stage which are preferably carried out in the same microplate plate in order to reduce as far as possible the incidence of experimental variability.

The calibration stage comprises the acquisition of the absorbance data relating to the samples at known increasing concentrations of defibrotide (standard), the statistical reprocessing of those data and the extrapolation of calibration curves, which express the correlation between the increase in the rate of the enzymatic reaction of the invention and the concentration of defibrotide present in the euglobulin fraction. In the measuring stage, owing to the correlation obtained in the calibration stage, it is possible to determine the unknown biological activities of samples of defibrotide on the basis of the absorbance values measured and processed under the same conditions.

In more detail, the experimental protocol generally provides for the preparation of several samples, both standard and unknown, at various known concentrations of defibrotide. The defibrotide samples are prepared by progressive dilution of the mother solutions in accordance with a predetermined dilution factor.

In the present method, it is preferred to prepare at least 5 concentrations of the standard and 5 concentrations of the sample to be tested, preparing at least 4 replicates for each concentration of the standard and, similarly, for each concentration of the test sample, generally for successive 1:2 dilutions of mother solutions.

Both the standard and test-sample concentrations of defibrotide are generally from 0.1 to 1000 μg/ml.

The concentrations of the test sample are preferably of the same order of magnitude as the concentrations of the standard.

In accordance with the above illustration, the measurements for each concentration are preferably carried out on 1 microplates where the position of each sample, the standard and the test sample, respectively, at corresponding concentration is preferably alternated. According to this scheme for the arrangement of the samples, which is explained in more detail in the experimental part, for each concentration of both standard and test-sample defibrotide, at least 4 absorbance values are measured for each time.

The set of measurements described above are taken at predetermined times, that is to say, first of all at time t_0, that is to say, when all of the components have been added, before the enzymatic reaction of the invention has started, and subsequently at precise intervals and for a period of time sufficient to acquire the necessary data.

Preferably, the absorbance measurements are continued up to a maximum of 90 minutes, with readings taken every 1-10 minutes. More advantageously, the readings are taken every minute. The photometric absorbance readings are performed at a wavelength which depends on the nature of the detectable group X freed in the course of the enzymatic hydrolytic reaction. In the specific case in which X is p-NA, the absorbance is measured at 405 nm.

The absorbance readings of the standard and unknown defibrotide samples, known as raw data, generally originate directly from the same apparatus that provides for the reading operation; they are tabulated in such a manner that an absorbance value is expressed for each time and well.

The raw data are then processed, using, for example, the Spread Sheet—Microsoft Excel®. This first processing operation leads to the calculation of the average absorbance and of the associated standard deviation, at each time and for each set of readings, each set comprising at least 4 replicates for each concentration of both standard and test-sample defibrotide.

Further statistical processing of the data is carried out with commercially available software for biological assay determination as described by Finney D J, Statistical Method in Biological Assay, 2nd ed. Ch. Griffin, London and relevant Pharmacopoeias.

To be more precise, according to the present invention, defibrotide biological assay determination can be performed using parallel line model, slope ration model and four-parameter logistic curve models as defined, for example, by the relevant European Pharmacopoeia General text 5.3, Statistical Analysis"

As illustrated in FIG. **1**, by placing the time on the abscissa and the absorbance on the ordinate, straight lines will be obtained whose slope "b" will be proportional to the rate of enzymatic reaction: by increasing the concentration of defibrotide, the rate of hydrolysis and, proportionally, the value of "b" will increase. Finally, the slope values, calculated as described above for each set of replicates of standard

defibrotide and test-sample defibrotide, are correlated with the concentration of defibrotide to which they relate. Suitable mathematical transformation of the abscissa (i.e. log of defibrotide concentration) can be used in lieu of the real value.

Graphically, that correlation gives rise to a sigmoid for the standard and a sigmoid for the test sample (FIG. **2**); the central portions of the sigmoid have two straight lines which are generally parallel and the distance between which is a function of the difference in biological activity between the test sample and the standard. This interval is used for potency determination using the parallel line model as described by Finney D J, Statistical Method in Biological Assay, 2nd ed. Ch. Griffin, London.

For a more specific determination, the four-parameter logistic curve models is used and in this case the entire sigmoid curve of both, sample and standard, is used for the calculation of the relative biological potency of the sample.

In a preferred embodiment of the present invention, the standard solutions and the solutions of the samples of defibrotide to be determined are introduced into the respective wells of the microplate. The euglobulin fraction are prepared at the moment of use and it is the dilution media of defibrotide stock solution. Finally, the solution containing the chromogenic substrate is added. The microplate is then placed in the thermostated reader and, after rapid agitation, readings of the system's absorbance are taken at predetermined intervals and for the predetermined period of time. The raw data obtained are then processed, thus determining the unknown activities of the defibrotide samples.

As it shall be appreciated from the following examples, the method according to the present invention allows to obtain liquid defibrotide formulations, preferably water solutions, having a defined biological activity and, in particular, having an activity of 25 to 35 IU/mg of defibrotide, preferably from 27.5 to 32.5 IU/mg and, more preferably, from 28 to 32 IU/mg.

Liquid defibrotide formulations are preferably marketed in form of containers, more preferably vials, containing 200 mg of defibrotide in 2.5 ml of buffered water solution (preferably at a pH from 6.5 to 8.5, more preferably from 7 to 8), to be diluted before use; consequently, when the biological activity is assessed with the method of the present invention, each container presents a defibrotide activity of 5000 to 7000 IU, preferably 5500 to 6500 IU, more preferably 5600 to 6400.

Those and other aspects of the invention will be better illustrated in the following examples which are not, however, to be regarded as limiting the invention.

EXAMPLES

The following materials were used in the examples given here:

Apparatus

Main features:	Microplate reader for UV-Vis absorbance determination equipped with thermostatic chamber and absorbance filter at 405 nm.		
Detection	Kinetic Absorbance determination at 405 nm		
Plate Type	96 · well clear for UV-Vis Determination		
Chamber Temperature	37° C.	Total Absorbance recording time	45-60 min
Absorbance recording frequency	About 1 × min		

Programs & Software

Microsoft Excel® (Microsoft Corporation, Redmond, Wash., USA)

Substances

Defibrotide (Gentium)

Chromogenic substrate S-2251 (Chromogenix Instrumentation Laboratory S.p.A., Milan, Italy)

Tris(hydroxymethyl)aminomethane (TRIS)-NaCl, (Sigma-Aldrich, Milan, Italy)

1N HCl (Carlo Erba reagenti, Milan, Italy)

IN NaOH (Carlo Erba reagenti, Milan, Italy)

Bovine Plasma (Tebu Bio Italia, Magenta (MI), Italy)

Glacial Acetic Acid (Carlo Erba reagenti, Milan, Italy)

Solutions

TRIS-NaCl (1 L Preparation): Into a 1 L beaker quantitatively transfer 6.06 g of TRIS-HCl and 2.2 g of NaCl. Dissolve in 500 mL of purified water and adjust the pH to 7.4 with about 40 mL of HCl 1N. Quantitatively transfer the solution into a volumetric flask of 1 L and dilute the solution to volume with purified water. Store the solution into refrigerator (2-8° C.)

Chromogenic Substrate S2251 (CAS 63589-93-5) 3 mM (15.2 mL Preparation): Dissolve about 25 mg of chromogenic substrate with 15.2 mL of purified water. Store the solution into refrigerator (2-8° C.)

Bovine euglobulins (10 mL Preparation). In a container with a minimum capacity of 300 mL introduce 240 mL of ice-cooled purified water and under stirring add 10 mL bovine plasma. Adjust the pH to 5.3±0.1 with acetic acid 1%. Allow to settle at 2-8° C. for 1 to 16 hours. Remove the clear supernatant solution by siphoning and collect the precipitate by centrifugation at 2.800 rpm for 5 minutes and 4° C. Suspend the precipitate dispersing mechanically (e.g., by means of a laboratory glass rod) in 5 mL of ice-cooled purified water, shake for about 5 min and collect the precipitate by centrifugation at 2.800 rpm for 5 minutes and 4° C. Disperse the precipitate mechanically into about 10 mL of TRIS-NaCl; to facilitate the dissolution of the precipitate crush the particles of the precipitate with a suitable instrument (es.: laboratory glass rod). Store the obtained suspension at 2-8° C. for not less than 1 hour and not more than 6 hours before its use.

Standard and Sample Defibrotide Solutions

Reference Stock Solution

Into 20 mL volumetric flask quantitatively transfer about 100 mg of defibrotide reference standard accurately weighed. Dissolve the powder with about 10 mL TRIS-NaCl and bring the volume with the same solvent. Dilute 1:4 the obtained solution with TRIS-NaCl in order to obtain a defibrotide RS solution of about 1.25 mg/mL.

Sample Stock Solution

Into 20 mL volumetric flask quantitatively transfer about 100 mg of defibrotide sample accurately weighed. Dissolve the powder with about 10 mL TRIS-NaCl and bring the volume with the same solvent. Dilute 1:4 the obtained solution with TRIS-NaCl in order to obtain a defibrotide sample solution of about 1.25 mg/mL.

Reference and Sample Solutions Preparation

a) Defibrotide 125 ug/mL: dilute 1:10 defibrotide stock solution (Reference and Sample) with TRIS NaCl (corresponding to 50 ug/mL into the plate well). Into an eppendorf tube quantitatively transfer 500 uL of the prepared solution and mix with 500 uL of euglobulin. Close the tube and store in ice-cooled water.

b) Defibrotide 62.5 ug/mL: dilute 1:2 solution (a) with TRIS NaCl (corresponding to 25 ug/mL into the plate well), Into an eppendorf tube quantitatively transfer 500 uL of the prepared solution and mix with 500 uL of euglobulin. Close the tube and store in ice-cooled water.

c) Defibrotide 31.25 ug/mL: dilute 1:2 solution (b) with TRIS NaCl (corresponding to 12.5 ug/mL into the plate well). Into an eppendorf tube quantitatively transfer 500 uL of the prepared solution and mix with 500 uL of euglobulin. Close the tube and store in ice-cooled water.

d) Defibrotide 12.5 ug/mL: dilute 1:2.5 solution (d) with TRIS NaCl (corresponding to 5 ug/mL into the plate well). Into an eppendorf tube quantitatively transfer 500 uL of the prepared solution and mix with 500 uL of euglobulin. Close the tube and store in ice-cooled water.

Blank Solution

Mix 1 volume of euglobulins with 1 volume of TRIS NaCl solution (ex.: 500 uL+500 uL)

Plate Deposition

According to the proposed deposition scheme (see Table 1 below) add in each well of the plate 200 uL of standard or sample or blank solution. Note different deposition scheme can be used according to the availability and configuration of automatic pipettes. However not less than 4 depositions for each reference and sample solution must be used for the assay.

Immediately before incubation of the plate into the microplate reader add in each well 50 uL of Chromogenic substrate.

TABLE 1

	1	2	3	4	5	6	7	8	9	10	11	12
A	—	BLK	S1_Ca	S1_Cb	S1_Cc	S1_Cd	—	—	—	—	—	—
B	—	BLK	S2_Ca	S2_Cb	S2_Cc	S2_Cd	—	—	—	—	—	—
C	—	BLK	S3_Ca	S3_Cb	S3_Cc	S3_Cd	—	—	—	—	—	—
D	—	BLK	S4_Ca	S4_Cb	S4_Cc	S4_Cd	—	—	—	—	—	—
E	—	BLK	U1_Ca	U1_Cb	U1_Cc	U1_Cd	—	—	—	—	—	—
F	—	BLK	U2_Ca	U2_Cb	U2_Cc	U2_Cd	—	—	—	—	—	—
G	—	BLK	U3_Ca	U3_Cb	U3_Cc	U3_Cd	—	—	—	—	—	—
H	—	BLK	U4_Ca	U4_Cb	U4_Cc	U4_Cd	—	—	—	—	—	—

S1, S2, S3, S4: Reference Solution deposition 1, deposition 2, deposition 3, deposition 4,
U1, U2, U2, U5: Sample solution deposition 1, deposition 2 deposition 3, deposition 4,
Ca, Cb, Cc etc.: Defibrotide reference and sample concentration a, b, c etc.
BLK Blank solution

Calculation and Results

From the kinetic plot "absorbance versus time" of the standard preparations (ex.: S1_Ca, S1_Cb, S1_Cc) identify a suitable linear range (ex.: from 30 to 35 min, see FIG. **3**).

Identification of the linear kinetic range (A@405 nm vs time).

Calculate for each preparation of the standard and of the sample the response of the assay (Slope) in the pre-defined time range as follow.

$$\text{Sample \& Standard Response} = \frac{A_b - A_a}{T_b - T_a} \times 1000$$

Where:

Aa is the Absorbance values at Time Ta (30 min from the plot above)

Ab is the Absorbance value at Time Tb (35 min from the plot above)

Report the obtained value in a tabular format as reported in table 2.

TABLE 2

Concentration Level	Standard Preparation				Sample Preparation			
[ug/mL]	S1	S2	S3	S4	U1	U2	U3	U4
5	S1_Cd	S2_Cd	S3_Cd	S4_Cd	U1_Cd	U2_Cd	U3_Cd	U4_Cd
12.5	S1_Cc	S2_Cc	S3_Cc	S4_Cc	U1_Cc	U2_Cc	U3_Cc	U4_Cc
25	S1_Cb	S2_Cb	S3_Cb	S4_Cb	U1_Cb	U2_Cb	U3_Cb	U4_Cb
50	S1_Ca	S2_Ca	S3_Ca	S4_Ca	U1_Ca	U2_Ca	U3_Ca	U4_Ca

Plot the responses for the substance to be examined and for the standard against the logarithms of the concentration and calculate the activity of the substance to be examined using the parallel line model as defined by the relevant 5.3.2 chapter of the Ph. Eur Current edition.

Not less than 3 consecutive serial dilutions of the reference and of the sample should be used (e.g., defibrotide concentration 5 μg/mL, 12.5 μg/mL, 25 μg/mL, 50 μg/mL, or 5 μg/mL, 12.5 μg/mL, 25 μg/mL, or 12.5 μg/mL, 25 μg/mL, 50 μg/mL).

Analysis of the Variance

The analysis of the variance is performed according to the section 5.3.2.3 of the Ph. Eur. current edition and Finney D J (1964) Statistical Method in Biological Assay 2nd ed.

Test for Validity

1) The linear regression terms is significant, i.e. the calculated probability is less than 0.05. If this criterion is not met, it is not possible to calculate 95% C.I.
2) The Term of non-parallelism is not significant, i.e. the calculated probability is less than 0.05.

3) The term for non linearity is not significant, i.e. the calculated probability is less than 0.05.

Acceptance Criteria

4) The estimated potency is not less than 90% and not more than 111% of the stated potency.

5) The confidence limits (P=0.95) of the estimated potency are not less than 80% and not more than 125% of the stated potency

Calculation

$$\text{Sample Potency } (UI/\text{mg}) = \frac{R \times \text{Std Potency } (UI/\text{mg}) \times Conc.Std\ (\text{mg/mL})}{Conc.Sample\ (\text{mg/mL})}$$

where:

R: Result of sample obtained by the parallel line model analysis

Std Potency: Stated potency of the Standard (UI/mg on dry substance)

Conc. Std: Concentration of the Standard (mg/mL on dry substance)

Conc. Sample: Concentration of the Sample (mg/mL on dry substance)

Assay Applied to Defibrotide Formulations

The above-disclosed assay has been used to determine the biological activity of liquid formulations containing 200 mg of defibrotide in 2.5 ml (80 mg per ml) and having the quali-quantitative composition reported in table 3.

TABLE 3

Component	Reference to Standard Quality	Function	200 mg Injection	Concentration per mL
Defibrotide	In-house standard	Drug Substance	200.00 mg	80.00 mg
Sodium Citrate, Dihydrate	USP-EP	Buffer	25.00 mg	10.00 mg
Water-for-injection	USP-EP	Solvent vehicle	q.s. to 2.5 mL	1.00 mL
Sodium hydroxide 1M or hydrochloric acid 1M	NF-EP	pH adjustment	q.s. to 6.8-7.8	—
Nitrogen	NF-EP	Inert gas to displace the air	q.s.	—

The results are reported in table 4.

TABLE 4

Batch	Potency IU/mg	Potency IU/container of 200 mg
688	31	6200
738	34	6800
785	32	6400
836	30	6000
0406	31	6200
DS0617	35	7000
DV0502*	*	Not available
DV0601	35	7000
1070010073	32	6400
1080010016	32	6400
1080020018	31	6200
1080030021	31	6200
1080040110	32	6400
1080050114	35	7000
1080060117	34	6800
Mean	33	
Min	30	
Max	35	
RSD (%)	5.4	

*Used as Reference Standard

The invention claimed is:

1. A method of treating Veno-Occlusive Disease (VOD) comprising administering to a patient in need thereof a defibrotide formulation consisting of defibrotide, sodium citrate, and water for injection, having a potency of 25 to 35 IU/mg, and a concentration of at least 80 mg/mL,

wherein the defibrotide potency is determined by a method comprising the steps of:

a) bringing into contact defibrotide, mammalian euglobulin and a substrate specific for plasmin which, by reaction with plasmin, provides a measurable product; and

b) measuring the amount of product formed at successive times, to thereby determine the potency of the defibrotide.

2. The method of claim **1**, wherein the formulation has a potency of 27.5 to 32.5 IU/mg.

3. The method of claim **2**, wherein the formulation has a potency of 28 to 32 IU/mg.

4. The method of claim **1**, wherein the formulation is a water solution.

5. The method of claim **4**, wherein the formulation has a pH of from 6.5 to 8.5.

6. The method of claim **5**, wherein the formulation has a pH of from 7 to 8.

7. The method of claim **1**, wherein the euglobulin is human, rabbit or bovine euglobulin.

8. The method of claim **1**, wherein plasmin which reacts with the substrate specific for plasmin is released by plasminogen contained in the mammalian euglobulin.

9. The method of claim **1**, wherein the substrate specific for the plasmin is a chromogenic substrate.

10. The method of claim **1**, wherein the substrate specific for the plasmin is a compound of formula A1-A2-A3-X in which A1 and A2 are non-polar amino acids, A3 is lysine or arginine and X is the measurable product.

11. The method of claim **10**, wherein the measurable product X is selected from the group consisting of para-nitroaniline and 2-naphthylamine.

12. The method of claim **10**, wherein the substrate specific for plasmin is H-D-Valyl-L-Leucyl-L-Lysine-p-nitroaniline.

13. The method of claim **10**, wherein the measurable product X is measured by spectrophotometry or spectrofluorimetry.

14. The method of claim **1**, wherein the mammalian euglobulin is obtained from a volume of plasma and reconstituted to the same volume of the originating plasma or diluted up to 1:10 with suitable buffer and the substrate specific for the plasmin is a chromogenic/fluorogenic substrate having a concentration of from 2.5 to 3.5 mM.

15. The method of claim **1**, wherein said method of determining the defibrotide potency is carried out in a reaction medium which is an aqueous solution buffered to a pH of from 7 to 8.

16. The method of claim **1**, wherein the method of determining the defibrotide potency is maintained at a temperature of from 35 to 39° C.

17. The method of claim **1**, wherein the substrate specific for plasmin has a concentration of from 0.3 to 4 mM.

18. The method of claim **1**, wherein the method of determining the defibrotide potency comprises the steps of: c) determining the rate of release of the measurable product during the course of the reaction of both a standard sample and a test sample of defibrotide; d) correlating the rate of release with the corresponding defibrotide concentration to obtain the potency of the test sample of defibrotide.

19. The method of claim **17**, wherein the concentration of the substrate specific for plasmin is 3 mM.

* * * * *

EXHIBIT C

US011746348B2

(12) **United States Patent**
Ignoni et al.

(10) **Patent No.: US 11,746,348 B2**
(45) **Date of Patent: *Sep. 5, 2023**

(54) **EUGLOBULIN-BASED METHOD FOR DETERMINING THE BIOLOGICAL ACTIVITY OF DEFIBROTIDE**

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This patent is subject to a terminal disclaimer.

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(58) **Field of Classification Search**

None

See application file for complete search history.

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(57) **ABSTRACT**

It is disclosed a method for determining the biological activity of defibrotide, which comprises the steps of: a) bringing into contact defibrotide, mammalian euglobulin and a substrate specific for the plasmin which, by reaction with the plasmin, provides a measurable product; and b) measuring the amount of product formed at successive times, to thereby determine the biological activity of the defibrotide. Liquid defibrotide formulations are also disclosed, preferably water solutions, having a defined biological activity and, in particular, having an activity of 25 to 35 IU/mg of defibrotide, preferably from 27 to 32 IU/mg and, more preferably, from 28 to 32 IU/mg.

19 Claims, 2 Drawing Sheets

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Figure 1

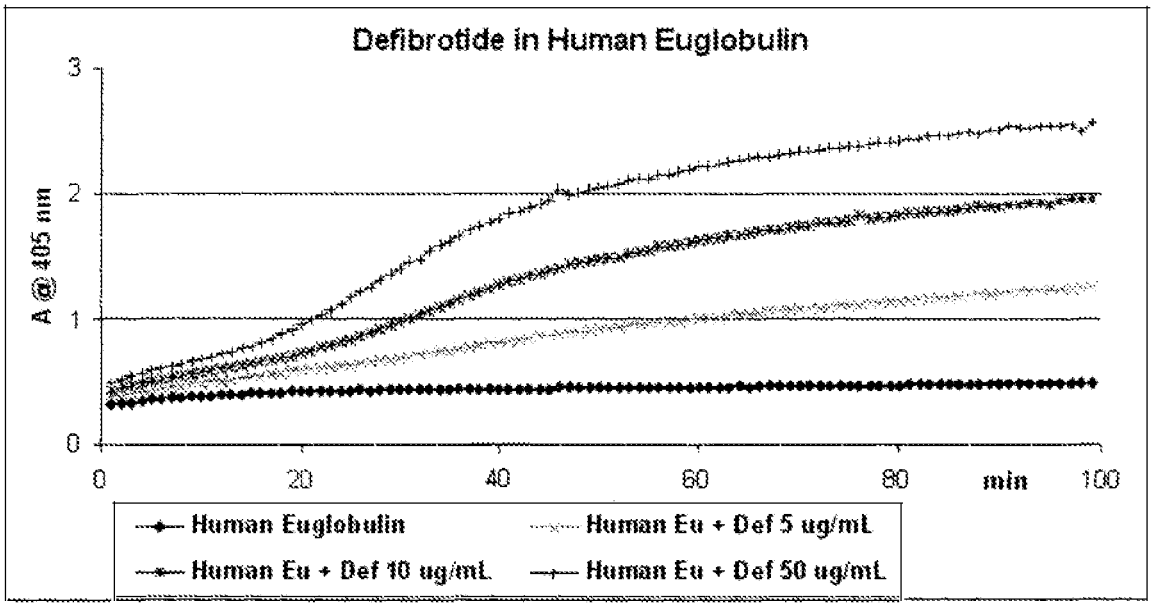


Figure 2

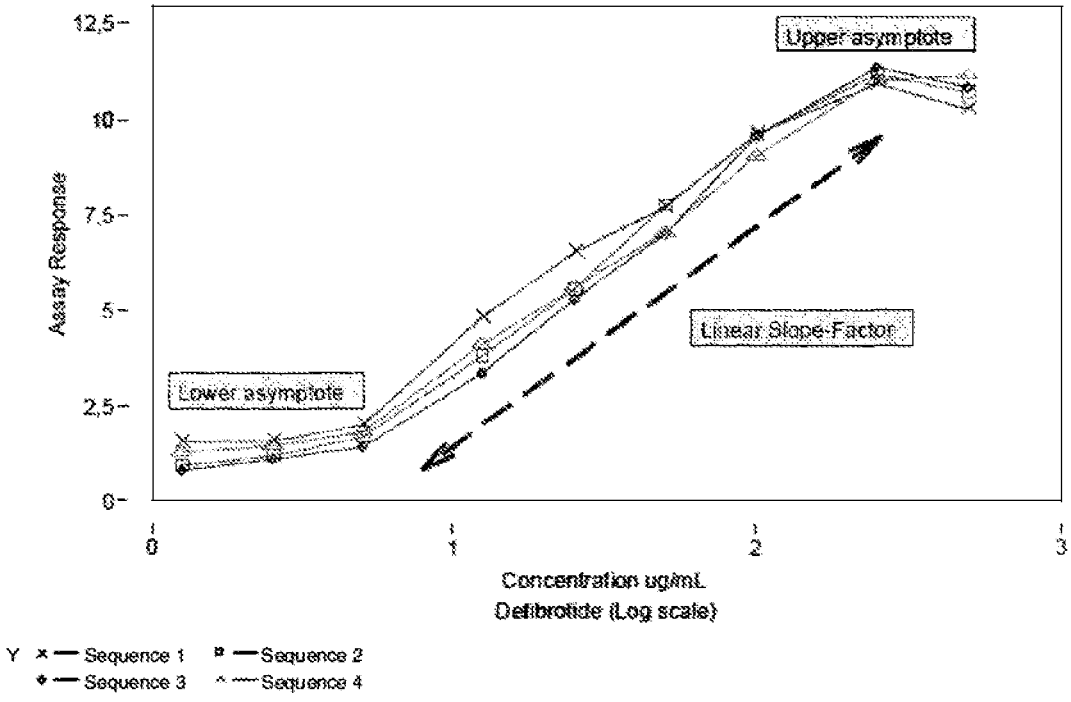
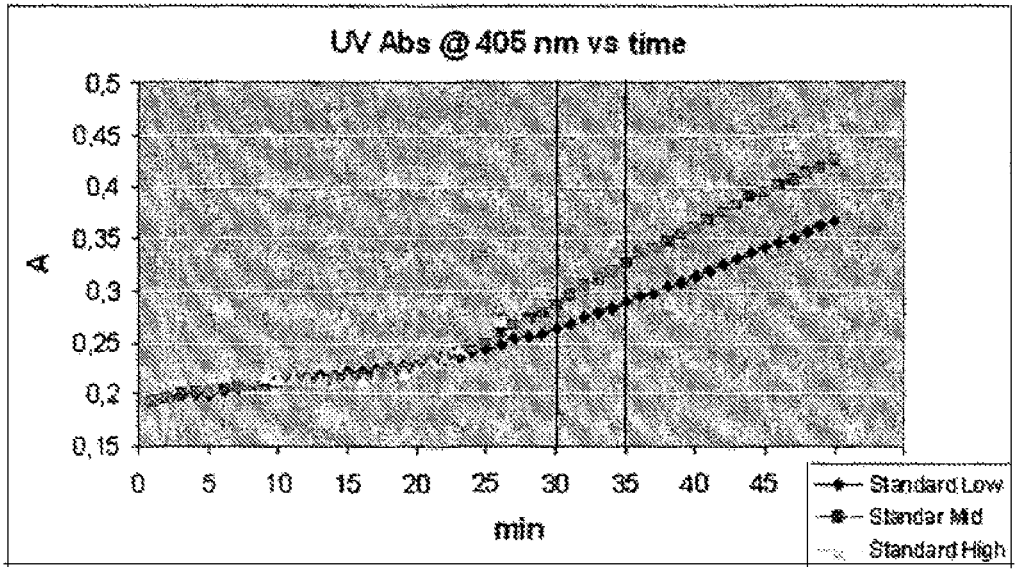


Figure 3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EUGLOBULIN-BASED METHOD FOR DETERMINING THE BIOLOGICAL ACTIVITY OF DEFIBROTIDE

CROSS-REFERENCE TO RELATED APPLICATIONS

This application is a continuation of U.S. application Ser. No. 16/816,741, filed Mar. 12, 2020, now U.S. Pat. No. 11,085,043, which is a continuation of U.S. application Ser. No. 15/844,801 filed Dec. 18, 2017, which is a continuation of U.S. application Ser. No. 14/408,272 filed Dec. 15, 2014, now U.S. Pat. No. 9,902,952, and which is a 371 of international PCT/IT2012/000193, filed Jun. 22, 2012, each of which is herein incorporated by reference in its entirety.

The present invention relates to a method for determining the biological activity of defibrotide and, more especially, relates to an indirect enzymatic method for determining the biological activity of defibrotide.

TECHNICAL FIELD OF THE INVENTION

Defibrotide (Merck Index, 1996, no. 2915) is a substance of natural origin which is obtained by extraction from animal organs and which is constituted by the sodium salt of polydeoxyribonucleotides having a low molecular weight. Defibrotide has been the subject of numerous pharmacological investigations which have suggested that it be applied in therapy as an anti-thrombotic agent (U.S. Pat. No. 3,829,567).

In addition, defibrotide has also been used successfully in the treatment of peripheral arteriopathies, in acute renal insufficiency (U.S. Pat. No. 4,694,134) or in acute myocardial ischaemia (U.S. Pat. No. 4,693,995).

Defibrotide is currently undergoing clinical trials to be used for the treatment and prevention of venous occlusive disease (VOD).

Like other biological substances which are obtained by extraction, defibrotide is also subject to a limited variability of composition which is typical of natural biopolymers. A classical example of this situation is provided by heparin whose variability from batch to batch in terms of chain length, molecular weight, composition, degree of sulphatation, etc. is well known. The consequence of this is that the same amounts by weight of defibrotide could in fact be non-equivalent from the point of view of a specific biological activity.

The process of extraction, isolation and purification cannot per se ensure absolute reproducibility of the product, precisely owing to its intrinsic biopolymeric nature.

However, if well controlled, it is possible to reduce this variability: for that purpose, studies have been made of standardized industrial processes for isolating defibrotide by extraction from organs, such as, for example, that described in U.S. Pat. No. 4,985,552.

The product obtained according to the above-mentioned process is characterized by the determination of some specific physico-chemical parameters, such as, for example, electrophoretic mobility, the coefficient of extinction, optical rotatory power and mass-average relative molecular mass. However, those parameters depend basically on the structure of defibrotide and are not capable of providing information on the biological activity thereof.

As far as we know, the only methods that have been reported to be used hitherto to evaluate the biological activity of defibrotide are the fibrin plate test and the thromboelastographic recording of the euglobulin lysis time

[Prino G., Mantovani M., Niada R., Coccheri S., Butti A., Indagini preliminari sull'attivita fibrinolitica, nell'animale e nell'uomo, di una nuova sostanza presente in diversi organi animali, Simposio Internazionale: La ricerca scientifica nell'industria farmaceutica in Italia, Rome, 2-4 Oct. 1975-11 Farmaco, Ed. Prat.) (1969), 24, 552-561] and the method based on plasmin disclosed in U.S. Pat. No. 7,338,777, herein incorporated by reference.

However, the above-mentioned method thromboelastographic recording of the euglobulin lysis time is characterized by considerable experimental complexity, by unsatisfactory reproducibility and precision and, in the specific case of thromboelastographic recording, by a response linearity limited to very restricted concentration ranges.

As to the plasmin-based method, the enzymatic activity of plasmin is normally determined by various standard in vitro tests. One of the most commonly used methods is the determination by spectrophotometry or fluorimetry of the chromogenic or fluorogenic compounds that are freed by the action of plasmin on suitable substrates [Haemostasis, (1978), 7, 138-145]. Peptide substrates having the formula A_1-A_2-A_3-X are generally used in which A_1 and A_2 are amino acids that are predominantly non-polar, A_3 is lysine or arginine and X represents the measurable freed compound, for example para-nitroaniline (pNa) or 2-naphthylamine (NA) [Haemostasis, (1978), 7, 146-149]. In addition to the above-mentioned peptide substrates, success has been achieved using other, simpler, compounds, such as, for example, p-nitrobenzyl-p-toluenesulphonyl-L-arginine [Haemostasis, (1978), 7, 105-108].

The rate at which the compound X is released into the incubation medium is proportional to the activity (Units/mg) of plasmin present in the sample. The method disclosed in U.S. Pat. No. 7,338,777 is thus based on the finding that, in the plasmin-evaluation tests described above, defibrotide increases the rate of release of compound X proportionally to its concentration.

However, such a method is conducted in TRIS buffer without any other plasma/serum activator/inhibitor. Therefore, the procedure does not reflect the physiological condition nor accurately simulates the mechanism of action of defibrotide in vivo.

Hitherto, therefore, no truly valid, precise and reproducible methods have been described and validated for determining the biological activity of defibrotide reflecting in an accurate way the mechanism of action of the product in a complex biological system (in vivo).

We have developed a simple and reliable method for determining the biological activity of defibrotide, which enables the samples obtained by extraction to be controlled and therefore enables medicinal preparations based on defibrotide to be standardized.

The method to which the present invention relates enables the specific biological activity of defibrotide to be determined in comparison with a reference standard with a high degree of precision and accuracy.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. **1** is a plot showing the kinetics of release of pNA from the substrate S-2251, by means of mammalian euglobulin fraction which is activated and non-activated with defibrotide (concentration 0-50 μg/ml, 0-100 min).

FIG. **2** is a plot illustrating the sigmoid that arises in relation to a standard and test sample of defibrotide.

FIG. **3** is a plot showing the "absorbance versus time" of the standard preparations (ex.: S1_Ca, S1_Cb, S1_Cc) identify a suitable linear range (ex.: from 30 to 35 min).

DESCRIPTION OF THE INVENTION

The present invention therefore relates to a method for determining the specific biological activity of samples of defibrotide, which method comprises the steps of:

a) bringing into contact defibrotide, euglobulin and a substrate specific for the plasmin which, by reaction with the plasmin, provides a measurable product and

b) measuring the amount of product formed at successive times.

The method of the invention is an indirect in vitro method for determining the activity of defibrotide, which is based on the functional interactions between defibrotide and euglobulin.

Euglobulin is that fraction of serum globulin that is insoluble in distilled water but soluble in saline solutions.

Euglobulin contains fibrinogen, PAI-1, tissue plasminogen activator (tPA), plasminogen, and to a lesser extent alpha 2-antiplasmin and also factor VIII.

The present inventors have surprisingly found that defibrotide catalyzes the hydrolysis of plasminogen into plasmin. Consequently, when defibrotide is incubated with euglobulin and a substrate specific for plasmin, such as a peptide of formula A_1-A_2-A_3-X as disclosed by Haemostasis, (1978), 7, 138-149, herein incorporated by reference, the rate at which the compound X is released into the incubation medium increases proportionally to the concentration of defibrotide itself.

In other terms, defibrotide catalyzes the hydrolysis of plasminogen contained in euglobulin into plasmin; which plasmin enzymatically reacts with the substrate specific for plasmin, preferably a cromogenic substrate, to provide a measurable product.

The method of the present invention thus further comprises the steps of: c) determining the rate of release of the measurable product during the course of the enzymatic reaction of both a standard sample and a test sample of defibrotide; d) correlating, mathematically and/or graphically, the rate of release with the corresponding defibrotide concentration to obtain the biological activity of the test sample of defibrotide.

The defibrotide sample used for the determination according to the present invention is generally prepared by extraction from organs in accordance with known procedures, such as described, for example, in U.S. Pat. No. 4,985,552 which has already been mentioned and which is also herein incorporated by reference.

A batch of normal industrially manufactured defibrotide was chosen as the reference sample (standard) and was used to prepare the calibration curves in accordance with the method of the present invention.

In general, the present method provides precise and accurate measurements of defibrotide even in the presence of contaminants, such as, for example, RNA, heparin, degraded defibrotide (defibrotide from which purin or pyrimidine has been removed) or ethanol, provided they are in concentrations, generally less than 10% by weight, such as not to impair the system.

In addition to permitting the determination of defibrotide, the method also allows the determination of other biologically equivalent substances derived from defibrotide, such as, for example, deaminated defibrotide or, more simply, defibrotide denatured/degraded by combination of physico-chemical conditions.

The present method is sufficiently sensitive to detect concentrations of defibrotide lower than or equal to 2.5 μg/ml (final concentration in the determination system) and, generally, expresses good correlation up to maximum concentration values higher than or equal to 1000 μg/ml.

The euglobulin used is generally any mammalian euglobulin fraction, such as, for example, bovine, porcine, rabbit 'or human euglobulin, with a preference for human and bovine euglobuline.

However, although euglobulin fraction is the enzymatic system of choice, the use of other equivalent enzymatic systems, such as, for example, diluted plasma and serum (up to 1:10 with buffers), artificially created or isolated combinations of plasminogen, tPA, uPA, PAI-1&2 alpha 2 antiplasmin and the like enzymatic systems which are chemically and biologically related and have similar functionality, falls within the scope of the present invention.

In the method of the present invention, the substrate for the plasmin may be understood as being any substrate specific for plasmin which, under the conditions of the method, frees a detectable hydrolysis product X.

Depending on the nature of the detectable group X, alternative systems of detection commonly known to the person skilled in the art can be adopted equally well. Spectrophotometric or fluorimetric detection systems are particularly advantageous, especially spectrophotometric systems.

The substrates generally used are ones that are specific for plasminoge-plasmin assay. It is preferable to use peptides of the formula A_1-A_2-A_3-X, in which A_1 and A_2 are amino acids that are predominantly non-polar, A_3 is lysine or arginine and X is the detectable group. Examples of those substrates are Val-Leu-Lys-pNa, Val-Phe-Lys-pNa or pyro-Glu-Phe-Lys-pNa in which the group X detectable by spectrophotometry is para-nitroaniline (pNA). Other suitable substrates, for example Val-Gly-Arg-2NA, contain 2-naphthylamine, which is measurable by fluorimetry. A particularly preferred substrate is the compound H-D-Valyl-L-Leucyl-L-Lysine-p-nitroaniline (H-D-Val-Leu-Lys-pNA).

The specific substrates used for determining defibrotide activity in euglobulin fraction are generally commercially available.

The determination method of the present invention is carried out by placing defibrotide sample in euglobulin solution, at a specific pH and molarity.

In particular, euglobulin fractions obtained from mammalian plasma are reconstituted dissolving and diluting the euglobulin to the original volume of the generating plasma with saline buffer (ex.: the quantity of euglobulin fraction obtained from 10 mL plasma are dissolved and reconstituted to 10 mL with saline buffer at pH between 7 and 8).

However, as regards of the substrate concentrations of from 0.3 to 4 mM, preferably from 2.5 to 3.5 mM and advantageously of 3 mM, are generally used in the case of a chromogenic substrate, while concentrations of from 0.05 to 0.15 mM are used in the case of a fluorogenic substrate.

The determination method of the invention, like other enzymatic methods, is sensitive to the pH of the medium.

In fact, it cannot generally be applied at extreme pH values where the enzymatic system would be inactivated.

It is also preferable for the pH of the medium not to undergo variation at any time during the period when measurements are being taken, and therefore euglobuline fraction is reconstituted with buffer systems selected from

those normally used for biological tests. Suitable buffer systems may be, for example, phosphate buffer, citrate buffer or tris(hydroxymethyl)aminomethane hydrochloride and sodium chloride (TRIS-NaCl) buffer. The reconstitution of the euglobuline fraction is preferably carried out with TRIS-NaCl.

In the present method it is usually preferred to maintain the pH of the medium in a range of approximately from 7 to 8, more preferably at approximately 7.4-7.6.

In addition, it is preferred to maintain the concentration of the buffer system in a range of from 10 to 200 mM, preferably at approximately 50 mM. More specifically for the TRIS-NaCl the concentrations should be 50 mM for TRIS and 150 mM for sodium chloride

The method of the invention for determining defibrotide biological activity, defibrotide sample solutions is diluted directly into euglobulin fraction, then the chromogenic or fluorogenic substrate is added. In particular, in order to enable the measurements it is preferable to preliminary dilute/dissolve defibrotide in TRIS-NaCl buffer in order to obtain a mother stock solution of both, sample and standard. From the mother stock solutions the sample and the standard are diluted, by serial dilution, into defined volume of euglobulin fraction till the analytical concentration range which is about 1 to 1000 μg/mL of defibrotide

An important parameter in the present method of determination is the temperature. It is preferable for the same temperature to be maintained throughout the entire duration of the measurements and for all of the samples determined, both as regards the construction of the reference curves and during the measuring stage. To that end, it is preferable to use temperature controlled apparatus and also, where necessary, it is possible to proceed with several sets of measurements, changing the position of the samples appropriately in order to ensure that the system has maximum thermal homogeneity.

Generally, this method of determination is applied in a temperature range of, for example, from 25 to 40° C., preferably from 35 to 39° C., and even more preferably at 37° C.

According to the present invention, measurement of the concentration of compound X released in the medium by the action of defibrotide starts when all of the reagents have been added and continues for a predetermined time and at a predetermined frequency as a function of the chemical nature of X and of the detection system.

Similarly to other methods of biological determination, the method of the invention also provides for a calibration stage and a measuring stage which are preferably carried out in the same microplateplate in order to reduce as far as possible the incidence of experimental variability.

The calibration stage comprises the acquisition of the absorbance data relating to the samples at known increasing concentrations of defibrotide (standard), the statistical reprocessing of those data and the extrapolation of calibration curves, which express the correlation between the increase in the rate of the enzymatic reaction of the invention and the concentration of defibrotide present in the euglobulin fraction. In the measuring stage, owing to the correlation obtained in the calibration stage, it is possible to determine the unknown biological activities of samples of defibrotide on the basis of the absorbance values measured and processed under the same conditions.

In more detail, the experimental protocol generally provides for the preparation of several samples, both standard and unknown, at various known concentrations of defibrotide. The defibrotide samples are prepared by progressive dilution of the mother solutions in accordance with a predetermined dilution factor.

In the present method, it is preferred to prepare at least 5 concentrations of the standard and 5 concentrations of the sample to be tested, preparing at least 4 replicates for each concentration of the standard and, similarly, for each concentration of the test sample, generally for successive 1:2 dilutions of mother solutions.

Both the standard and test-sample concentrations of defibrotide are generally from 0.1 to 1000 μg/ml.

The concentrations of the test sample are preferably of the same order of magnitude as the concentrations of the standard.

In accordance with the above illustration, the measurements for each concentration are preferably carried out on 1 microplates where the position of each sample, the standard and the test sample, respectively, at corresponding concentration is preferably alternated. According to this scheme for the arrangement of the samples, which is explained in more detail in the experimental part, for each concentration of both standard and test-sample defibrotide, at least 4 absorbance values are measured for each time.

The set of measurements described above are taken at predetermined times, that is to say, first of all at time to, that is to say, when all of the components have been added, before the enzymatic reaction of the invention has started, and subsequently at precise intervals and for a period of time sufficient to acquire the necessary data.

Preferably, the absorbance measurements are continued up to a maximum of 90 minutes, with readings taken every 1-10 minutes. More advantageously, the readings are taken every minute. The photometric absorbance readings are performed at a wavelength which depends on the nature of the detectable group X freed in the course of the enzymatic hydrolytic reaction. In the specific case in which X is p-NA, the absorbance is measured at 405 nm.

The absorbance readings of the standard and unknown defibrotide samples, known as raw data, generally originate directly from the same apparatus that provides for the reading operation; they are tabulated in such a manner that an absorbance value is expressed for each time and well.

The raw data are then processed, using, for example, the Spread Sheet—Microsoft Excel®. This first processing operation leads to the calculation of the average absorbance and of the associated standard deviation, at each time and for each set of readings, each set comprising at least 4 replicates for each concentration of both standard and test-sample defibrotide.

Further statistical processing of the data is carried out with commercially available software for biological assay determination as described by Finney D J, Statistical Method in Biological Assay, 2nd ed. Ch. Griffin, London and relevant Pharmacopoeias.

To be more precise, according to the present invention, defibrotide biological assay determination can be performed using parallel line model, slope ration model and four-parameter logistic curve models as defined, for example, by the relevant European Pharmacopoeia General text 5.3, Statistical Analysis"

As illustrated in FIG. **1**, by placing the time on the abscissa and the absorbance on the ordinate, straight lines will be obtained whose slope "b" will be proportional to the rate of enzymatic reaction: by increasing the concentration of defibrotide, the rate of hydrolysis and, proportionally, the value of "b" will increase. Finally, the slope values, calculated as described above for each set of replicates of standard

defibrotide and test-sample defibrotide, are correlated with the concentration of defibrotide to which they relate. Suitable mathematical transformation of the abscissa (i.e. log of defibrotide concentration) can be used in lieu of the real value.

Graphically, that correlation gives rise to a sigmoid for the standard and a sigmoid for the test sample (FIG. **2**); the central portions of the sigmoid have two straight lines which are generally parallel and the distance between which is a function of the difference in biological activity between the test sample and the standard. This interval is used for potency determination using the parallel line model as described by Finney D J, Statistical Method in Biological Assay, 2nd ed. Ch. Griffin, London.

For a more specific determination, the four-parameter logistic curve models is used and in this case the entire sigmoid curve of both, sample and standard, is used for the calculation of the relative biological potency of the sample.

In a preferred embodiment of the present invention, the standard solutions and the solutions of the samples of defibrotide to be determined are introduced into the respective wells of the microplate. The euglobulin fraction are prepared at the moment of use and it is the dilution media of defibrotide stock solution. Finally, the solution containing the chromogenic substrate is added. The microplate is then placed in the thermostated reader and, after rapid agitation, readings of the system's absorbance are taken at predetermined intervals and for the predetermined period of time. The raw data obtained are then processed, thus determining the unknown activities of the defibrotide samples.

As it shall be appreciated from the following examples, the method according to the present invention allows to obtain liquid defibrotide formulations, preferably water solutions, having a defined biological activity and, in particular, having an activity of 25 to 35 IU/mg of defibrotide, preferably from 27.5 to 32.5 IU/mg and, more preferably, from 28 to 32 IU/mg.

Liquid defibrotide formulations are preferably marketed in form of containers, more preferably vials, containing 200 mg of defibrotide in 2.5 ml of buffered water solution (preferably at a pH from 6.5 to 8.5, more preferably from 7 to 8), to be diluted before use; consequently, when the biological activity is assessed with the method of the present invention, each container presents a defibrotide activity of 5000 to 7000 IU, preferably 5500 to 6500 IU, more preferably 5600 to 6400.

Those and other aspects of the invention will be better illustrated in the following examples which are not, however, to be regarded as limiting the invention.

EXAMPLES

The following materials were used in the examples given here:

Apparatus

Main features:	Microplate reader for UV-Vis absorbance determination equipped with thermostatic chamber and absorbance filter at 405 nm.		
Detection	Kinetic Absorbance determination at 405 nm		
Plate Type	96-well clear for UV-Vis Determination		
Chamber Temperature	37° C.	Total Absorbance recording time	45-60 min
Absorbance recording frequency	About 1 × min		

Programs & Software

Microsoft Excel® (Microsoft Corporation, Redmond, Wash., USA)

Substances

Defibrotide (Gentium)

Chromogenic substrate S-2251 (Chromogenix Instrumentation Laboratory S.p.A., Milan, Italy)

Tris(hydroxymethyl)aminomethane (TRIS)-NaCl, (Sigma-Aldrich, Milan, Italy)

1N HCl (Carlo Erba reagenti, Milan, Italy)

1N NaOH (Carlo Erba reagenti, Milan, Italy)

Bovine Plasma (Tebu Bio Italia, Magenta (MI), Italy)

Glacial Acetic Acid (Carlo Erba reagenti, Milan, Italy)

Solutions

TRIS-NaCl (1 L Preparation): Into a 1 L beaker quantitatively transfer 6.06 g of TRIS-HCl and 2.2 g of NaCl. Dissolve in 500 mL of purified water and adjust the pH to 7.4 with about 40 mL of HCl 1N. Quantitatively transfer the solution into a volumetric flask of 1 L and dilute the solution to volume with purified water. Store the solution into refrigerator (2-8° C.)

Chromogenic Substrate S2251 (CAS 63589-93-5) 3 mM (15.2 mL Preparation): Dissolve about 25 mg of chromogenic substrate with 15.2 mL of purified water. Store the solution into refrigerator (2-8° C.)

Bovine euglobulins (10 mL Preparation). In a container with a minimum capacity of 300 mL introduce 240 mL of ice-cooled purified water and under stirring add 10 mL bovine plasma. Adjust the pH to 5.3±0.1 with acetic acid 1%. Allow to settle at 2-8° C. for 1 to 16 hours. Remove the clear supernatant solution by siphoning and collect the precipitate by centrifugation at 2.800 rpm for 5 minutes and 4° C. Suspend the precipitate dispersing mechanically (e.g.: by means of a laboratory glass rod) in 5 mL of ice-cooled purified water, shake for about 5 min and collect the precipitate by centrifugation at 2.800 rpm for 5 minutes and 4° C. Disperse the precipitate mechanically into about 10 mL of TRIS-NaCl; to facilitate the dissolution of the precipitate crush the particles of the precipitate with a suitable instrument (es.: laboratory glass rod). Store the obtained suspension at 2-8° C. for not less than 1 hour and not more than 6 hours before its use.

Standard and Sample Defibrotide Solutions

Reference Stock Solution

Into 20 mL volumetric flask quantitatively transfer about 100 mg of defibrotide reference standard accurately weighed. Dissolve the powder with about 10 mL TRIS-NaCl and bring the volume with the same solvent. Dilute 1:4 the obtained solution with TRIS-NaCl in order to obtain a defibrotide RS solution of about 1.25 mg/mL.

Sample Stock Solution

Into 20 mL volumetric flask quantitatively transfer about 100 mg of defibrotide sample accurately weighed. Dissolve the powder with about 10 mL TRIS-NaCl and bring the volume with the same solvent. Dilute 1:4 the obtained solution with TRIS-NaCl in order to obtain a defibrotide sample solution of about 1.25 mg/mL.

Reference and Sample Solutions Preparation

a) Defibrotide 125 ug/mL: dilute 1:10 defibrotide stock solution (Reference and Sample) with TRIS NaCl (corresponding to 50 ug/mL into the plate well). Into an eppendorf tube quantitatively transfer 500 uL of the prepared solution and mix with 500 uL of euglobulin. Close the tube and store in ice-cooled water.

b) Defibrotide 62.5 ug/mL: dilute 1:2 solution (a) with TRIS NaCl (corresponding to 25 ug/mL into the plate well), Into an eppendorf tube quantitatively transfer 500 uL of the prepared solution and mix with 500 uL of euglobulin. Close the tube and store in ice-cooled water.

c) Defibrotide 31.25 ug/mL: dilute 1:2 solution (b) with TRIS NaCl (corresponding to 12.5 ug/mL into the plate well). Into an eppendorf tube quantitatively transfer 500 uL of the prepared solution and mix with 500 uL of euglobulin. Close the tube and store in ice-cooled water.

d) Defibrotide 12.5 ug/mL: dilute 1:2.5 solution (d) with TRIS NaCl (corresponding to 5 ug/mL into the plate well). Into an eppendorf tube quantitatively transfer 500 uL of the prepared solution and mix with 500 uL of euglobulin. Close the tube and store in ice-cooled water.

Blank Solution

Mix 1 volume of euglobulins with 1 volume of TRIS NaCl solution (ex.: 500 uL+500 uL)

Plate Deposition

According to the proposed deposition scheme (see Table 1 below) add in each well of the plate 200 uL of standard or sample or blank solution. Note different deposition scheme can be used according to the availability and configuration of automatic pipettes. However not less than 4 depositions for each reference and sample solution must be used for the assay.

Immediately before incubation of the plate into the microplate reader add in each well 50 uL of Chromogenic substrate.

TABLE 1

	1	2	3	4	5	6	7	8	9	10	11	12
A	—	BLK	S1_Ca	S1_Cb	S1_Cc	S1_Cd	—	—	—	—	—	—
B	—	BLK	S2_Ca	S2_Cb	S2_Cc	S2_Cd	—	—	—	—	—	—
C	—	BLK	S3_Ca	S3_Cb	S3_Cc	S3_Cd	—	—	—	—	—	—
D	—	BLK	S4_Ca	S4_Cb	S4_Cc	S4_Cd	—	—	—	—	—	—
E	—	BLK	U1_Ca	U1_Cb	U1_Cc	U1_Cd	—	—	—	—	—	—
F	—	BLK	U2_Ca	U2_Cb	U2_Cc	U2_Cd	—	—	—	—	—	—
G	—	BLK	U3_Ca	U3_Cb	U3_Cc	U3_Cd	—	—	—	—	—	—
H	—	BLK	U4_Ca	U4_Cb	U4_Cc	U4_Cd	—	—	—	—	—	—

S1, S2, S3, S4: Reference Solution deposition 1, deposition 2, deposition 3, deposition 4, U1, U2, U2, U5: Sample solution deposition 1, deposition 2 deposition 3, deposition 4, Ca, Cb, Cc etc.: Defibrotide reference and sample concentration a, b, c etc.

BLK Blank Solution

Calculation and Results

From the kinetic plot "absorbance versus time" of the standard preparations (ex.: S1_Ca, S1_Cb, S1_Cc) identify a suitable linear range (ex.: from 30 to 35 min, see FIG. **3**).

Identification of the linear kinetic range (A@405 nm vs time).

Calculate for each preparation of the standard and of the sample the response of the assay (Slope) in the pre-defined time range as follow.

$$\text{Sample \& Standard Response} = \frac{A_b - A_a}{T_b - T_a} \times 1000$$

Where:

Aa is the Absorbance values at Time Ta (30 min from the plot above)

Ab is the Absorbance value at Time Tb (35 min from the plot above)

Report the obtained value in a tabular format as reported in table 2.

TABLE 2

Concentration Level	Standard Preparation				Sample Preparation			
[ug/mL]	S1	S2	S3	S4	U1	U2	U3	U4
5	S1_Cd	S2_Cd	S3_Cd	S4_Cd	U1_Cd	U2_Cd	U3_Cd	U4_Cd
12.5	S1_Cc	S2_Cc	S3_Cc	S4_Cc	U1_Cc	U2_Cc	U3_Cc	U4_Cc
25	S1_Cb	S2_Cb	S3_Cb	S4_Cb	U1_Cb	U2_Cb	U3_Cb	U4_Cb
50	S1_Ca	S2_Ca	S3_Ca	S4_Ca	U1_Ca	U2_Ca	U3_Ca	U4_Ca

Plot the responses for the substance to be examined and for the standard against the logarithms of the concentration and calculate the activity of the substance to be examined using the parallel line model as defined by the relevant 5.3.2 chapter of the Ph. Eur Current edition. Not less than 3 consecutive serial dilutions of the reference and of the sample should be used (e.g., defibrotide concentration 5 μg/mL, 12.5 μg/mL, 25 μg/mL, 50 μg/mL, or 5 μg/mL, 12.5 μg/mL, 25 μg/mL, or 12.5 μg/mL, 25 μg/mL, 50 μg/mL).

Analysis of the Variance

The analysis of the variance is performed according to the section 5.3.2.3 of the Ph. Eur. current edition and Finney D J (1964) Statistical Method in Biological Assay 2nd ed.

Test for Validity

1) The linear regression terms is significant, i.e. the calculated probability is less than 0.05. If this criterion is not met, it is not possible to calculate 95% C.I.

2) The Term of non-parallelism is not significant, i.e. the calculated probability is less than 0.05.

3) The term for non linearity is not significant, i.e. the calculated probability is less than 0.05.

Acceptance Criteria

4) The estimated potency is not less than 90% and not more than 111% of the stated potency.

5) The confidence limits (P=0.95) of the estimated potency are not less than 80% and not more than 125% of the stated potency

Calculation

$$\text{Sample Potency } (UI/\text{mg}) = \frac{R \times \text{Std Potency } (UI/\text{mg}) \times \text{Conc.Std (mg/mL)}}{\text{Conc.Sample (mg/mL)}}$$

where:

R: Result of sample obtained by the parallel line model analysis

Std Potency: Stated potency of the Standard (UI/mg on dry substance)

Conc. Std: Concentration of the Standard (mg/mL on dry substance)

Conc. Sample: Concentration of the Sample (mg/mL on dry substance)

Assay Applied to Defibrotide Formulations

The above-disclosed assay has been used to determine the biological activity of liquid formulations containing 200 mg of defibrotide in 2.5 ml (80 mg per ml) and having the quali-quantitative composition reported in table 3.

TABLE 3

Component	Reference to Standard Quality	Function	200 mg Injection	Concentration per mL
Defibrotide	In-house standard	Drug Substance	200.00 mg	80.00 mg
Sodium Citrate, Dihydrate	USP-EP	Buffer	25.00 mg	10.00 mg
Water-for-injection	USP-EP	Solvent/ vehicle	q.s. to 2.5 mL	1.00 mL
Sodium hydroxide 1M or hydrochloric acid 1M	NF-EP	pH adjustment	q.s. to 6.8-7.8	—
Nitrogen	NF-EP	Inert gas to displace the air	q.s.	—

The results are reported in table 4.

TABLE 4

Batch	Potency IU/mg	Potency IU/container of 200 mg
688	31	6200
738	34	6800
785	32	6400
836	30	6000
0406	31	6200
DS0617	35	7000
DV0502*	*	Not available
DV0601	35	7000
1070010073	32	6400
1080010016	32	6400
1080020018	31	6200
1080030021	31	6200
1080040110	32	6400
1080050114	35	7000
1080060117	34	6800
Mean	33	
Min	30	
Max	35	
RSD (%)	5, 4	

*Used as Reference Standard

The invention claimed is:

1. A defibrotide formulation consisting of defibrotide, sodium citrate, and water for injection, having a potency of 25 to 35 IU/mg, and a concentration of at least 80 mg/mL, wherein the defibrotide potency is determined by a method comprising the steps of:

a) bringing into contact defibrotide, a biological composition comprising plasminogen, and a substrate specific for plasmin which, by reaction with plasmin, provides a measurable product; and

b) measuring the amount of product formed at successive times, to thereby determine the potency of the defibrotide.

2. The defibrotide formulation of claim **1**, wherein the formulation has a potency of 27.5 to 32.5 IU/mg.

3. The defibrotide formulation of claim **2**, wherein the formulation has a potency of 28 to 32 IU/mg.

4. The defibrotide formulation of claim **1**, wherein the formulation is a water solution.

5. The defibrotide formulation of claim **4**, wherein the formulation has a pH of from 6.5 to 8.5.

6. The defibrotide formulation of claim **5**, wherein the formulation has a pH of from 7 to 8.

7. The defibrotide formulation of claim **1**, wherein the biological composition comprising plasminogen comprises a human, rabbit or bovine plasminogen.

8. The defibrotide formulation of claim **1**, wherein plasmin which reacts with the substrate specific for plasmin is released by the plasminogen.

9. The liquid defibrotide formulation of claim **1**, wherein the substrate specific for the plasmin is a chromogenic substrate.

10. The defibrotide formulation of claim **1**, wherein the substrate specific for the plasmin is a compound of formula A1-A2-A3-X in which A1 and A2 are non-polar amino acids, A3 is lysine or arginine and X is the measurable product.

11. The defibrotide formulation of claim **10**, wherein the measurable product X is selected from the group consisting of para-nitroaniline and 2-naphthylamine.

12. The defibrotide formulation of claim **10**, wherein the substrate specific for plasmin is H-D-Valyl-L-Leucyl-L-Lysine-p-nitroaniline.

13. The defibrotide formulation of claim **10**, wherein the measurable product X is measured by spectrophotometry or spectrofluorimetry.

14. The defibrotide formulation of claim **1**, wherein the biological composition comprising plasminogen is obtained from a volume of plasma and reconstituted to the same volume of the originating plasma or diluted up to 1:10 with suitable buffer and the substrate specific for the plasmin is a chromogenic/fluorogenic substrate having a concentration of from 2.5 to 3.5 mM.

15. The defibrotide formulation of claim **1**, wherein said method is carried out in a reaction medium which is an aqueous solution buffered to a pH of from 7 to 8.

16. The defibrotide formulation of claim **1**, wherein the method is maintained at a temperature of from 35 to 39° C.

17. The defibrotide formulation of claim **1**, wherein the substrate specific for plasmin has a concentration of from 0.3 to 4 mM.

18. The defibrotide formulation of claim **1**, wherein the method comprises the steps of: c) determining the rate of release of the measurable product during the course of the reaction of both a standard sample and a test sample of defibrotide; d) correlating the rate of release with the corresponding defibrotide concentration to obtain the potency of the test sample of defibrotide.

19. The defibrotide formulation of claim **17**, wherein the concentration of the substrate specific for plasmin is 3 mM.

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